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A COMPARISON OF THE BACTERIOSTATIC ACTIVITIES OF SOME OF THE NEWER SULFONAMIDE COMPOUNDS[1]

ELEANOR A. BLISS AND HELEN C. DEITZ

From the Department of Preventive Medicine, The Johns Hopkins University

Four diazine derivatives of sulfanilamide are receiving considerable attention in the clinic (1) but while reports have appeared in which one or two of them have been compared with the parent compound, sulfadiazine, no study dealing with all four simultaneously has yet come out. We have, therefore, undertaken such a comparison. In the present paper the results of *in vitro* tests with sulfadiazine, sulfapyrazine, sulfamerazine and sulfamethazine are given. In addition to these, three other sulfonamides, dimethylacroyl sulfanilamide, dimethylbenzoyl sulfanilamide and homosulfanilamide were studied and the results are included. An examination of the therapeutic properties of the first six of these compounds in experimental infections in mice is in progress.

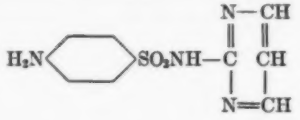
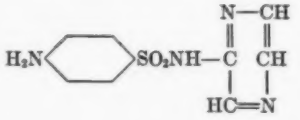
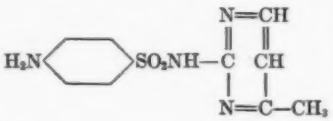
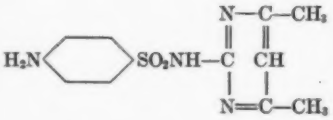
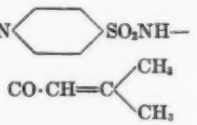
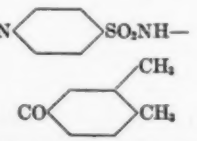
Certain properties of the compounds are given in Table I. The diazine derivatives are well known (2, 3, 4, 5, 6, 7, 8, 9, 10, 11) and need not be described at this time. The other three compounds, however, have not been widely used in this country, so a word about them is in order. The acroyl derivative of sulfanilamide was first prepared in Switzerland where it bears the trade name of Irgamide. Its acute toxicity for mice was found to be much lower than that of sulfapyridine and sulfathiazole. In experimental pneumococcal and streptococcal infections in mice, Högger (12) found it to be as effective as sulfapyridine. Markoff (13) and Scherer (14) have reported favorably upon its clinical use. Dimethylbenzoyl sulfanilamide, another product of Switzerland, is known there as Irgafen. Less direct information is at hand concerning it, because of the interruption in the receipt of foreign journals. Gsell (15) and Markoff (16) are quoted as having found it useful in the clinic. The manufacturers[2] state that sodium Irgafen is "no more toxic for mice than the sodium salts of sulfapyridine

[1] Part of the work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Johns Hopkins University.

[2] J. R. Geigy, S. A., Basle, Switzerland.

TABLE I

Properties of the compounds

NAME	STRUCTURAL FORMULA	MOL. WT.	SOLUBILITY AT 37°C. (MGM./100 CC.)	
			Water	Urine—pH 7
Sulfadiazine 2-sulfanilamido pyrimidine		250	12.3 (2)	80 (9)
Sulfapyrazine 2-sulfanilamido pyrazine		250	5.2 (4)	
Sulfamerazine 2-sulfanilamido 4-methylpyrimidine		264	31.8 (2)	100 (9)
Sulfamethazine 2-sulfanilamido 4,6-dimethylpyrimidine		278	250 (7)* approx	
Irgamide N_1 dimethylacroyl sulfanilamide		254	slight	320 (mfg)
Irgafen N_1-3,4-dimethylbenzoyl sulfanilamide		297	slight	277 (mfg)

* Gilligan and Plummer (32) have pointed out that this value is for the hemi-hydrate. They, however, do not give the solubility of true sulfamethazine in mgm. per 100 cc. but in sulfadiazine equivalents.

TABLE I—*Continued*

NAME	STRUCTURAL FORMULA	MOL. WT.	SOLUBILITY AT 37°C. (MGM./100 CC.)	
			Water	Urine—pH 7
Homosulfanilamide (Marfanil) 4-aminomethylbenzene sulfonamide		186	high 10,000+	
Sulfanilamide		172	1480	

and sulfathiazole," that it produces high blood levels readily and that it is excreted more slowly than sulfadiazine. Both Irgamide and Irgafen are only slightly soluble in water but in urine at pH 7 the solubility of the first compound is said to be 320 mgm. per 100 cc. and that of the second 277 mgm. per 100 cc. The manufacturers also emphasize the fact that the sodium salts of both drugs are stable at a lower pH than are the other sulfonamides.

Homosulfanilamide, or 4-aminomethyl benzenesulfonamide, appears to have been synthesized in Germany in 1938 or 39, but the first reference to it which has come to hand is that of Guleke, 1940 (17). For two or three years it was referred to as a secret compound (18) but its formula and properties were made public by Klarer (19) late in 1941. Meanwhile, however, Miller and his associates (20) reported on its method of preparation and Domagk (21) and Schreus (18, 22) had engaged in a polemic concerning its effect on experimental gas gangrene. While numerous reports of its use have appeared in the German literature, it has been overlooked here until quite recently when it was mentioned by McKee, Hamre and Rake (23). Our interest in it was aroused by Major Champ Lyons who was in touch with the work of McKee and her associates. It was suggested by the Subcommittee on Surgical Infections of the Committee on Medical Research that we study this compound—particularly its reputed (21, 23) insusceptibility to PABA antagonism. This phase of our investigations has been reported elsewhere (24) and will be published in full later on.

In Germany, this compound was originally called Mesudin but is now known in Europe as Marfanil. The term "homosulfanilamide" was arrived at, according to Dr. Rake, by analogy with "homocystine," (homosulfanilamide bears the same relationship to sulfanilamide that homocystine does to cystine—that is, it differs by just one CH_2 group). Other investigators (25), however, have a fourth name for it—sulfamylon. The present authors prefer the brief trade name Marfanil, but realize that it has no chemical significance.

EXPERIMENTAL DATA

Media. Various media were employed: PDW (peptone-dextrose-water, White (26)) for the staphylococci, Group D β haemolytic streptococci and E. coli; S1G1 (a synthetic medium (27)) also for E. coli, and BPD (PDW plus 1% defibrinated rabbit's blood) for the Group A haemolytic streptococcus and the pneumococcus.

Drugs. The sodium salts of all of the compounds except sulfanilamide and homosulfanilamide were used. Even so, the higher concentrations were prepared with difficulty. Sulfapyrazine and sulfamerazine came out of solution in the broth media at concentrations above 200–300 mgm. per 100 cc., sulfadiazine above 500 mgm. per 100 cc. and sulfamethazine above 600 mgm. per 100 cc. Irgamide and Irgafen were stable up to 1,000 mgm. per 100 cc. but in blood-PDW Irgafen at 500 mgm. per 100 cc. gave rise to a milky turbidity. This was not seen in the absence of blood.

General Procedure. The tests were run in Wassermann tubes. In all cases the final drug-medium volume was 5 cc. to which was added the inoculum of 0.1 cc. of diluted culture. The same medium which was used in the tests was used in growing and diluting the cultures. The number of organisms in the inoculum varied but was of course constant in any given test. Incubation was at 37°C. Growth, as shown by visible turbidity, was observed at 24 hours and, in certain tests, daily for 5 days.

Antibacteriostatic Tests. One series of tests was run as follows: To constant drug concentrations in S1G1 medium, decreasing amounts of p-amino benzoic acid (PABA) were added. The solutions were prepared on a molecular basis. The final volume of the mixtures was 5 cc. and this was inoculated with 0.1 cc. of a 1:1,000 dilution of the

test organism—E. coli. Growth was observed for 5 days. The number of molecules of drug which are required to neutralize one molecule of PABA was determined by this method.

In a second series of experiments a constant amount—1 mgm. per 100 cc.—of PABA was run against serial two-fold dilutions of the drugs in the synthetic medium. The starting point for the dilution was 81.96 mgm. per 100 cc. for the less soluble compounds and 655.36 mgm. per 100 cc. for the others. These concentrations approximate $\frac{2^{13}}{100}$ and $\frac{2^{16}}{100}$ mgm. per 100 cc. respectively. White, Litchfield and Marshall (28) used this dilution scale in measuring quantitatively the bacteriostatic activity of four sulfonamides in different media. In the present study the tests were made in regular test tubes but the final drug-medium volume was again 5 cc. and the inoculum 0.1 cc. of a 1:10,000 dilution of E. coli. In this way the relative anti-PABA action of the compounds was determined on a weight basis.

RESULTS

The results are shown in the tables. They are expressed, except in the first antibacteriostatic tests, as the minimal inhibitory concentrations, that is, the smallest amounts of the drugs, in milligrams per 100 cc., which prevented visible growth at 24 hours and at 2 days or 5 days. When more than one test was run, the mean has been estimated and the range shown. In the first series of antibacteriostatic tests the results are recorded as the smallest amount of PABA which permitted full growth at 24 hours and at 5 days. The last column in Tables II–VI inclusive gives the "activity ratios" (Marshall et al—29) of the compounds, using sulfanilamide as the standard of comparison. The figure is arrived at by dividing the 24 hour minimal inhibitory concentration of sulfanilamide by that of the drug.

In Table II it is seen that homosulfanilamide showed the greatest activity against the Group A haemolytic streptococcus in the blood-peptone-dextrose-water medium. Irgamide and sulfanilamide were equally inactive. Sulfapyrazine was the most potent of the diazine derivatives but its effect was equalled by that of Irgafen.

Table III shows that, of the four compounds tested, only homosulfanilamide had an appreciable effect upon the Group D haemolytic

streptococcus. It is well known that these organisms are resistant to the sulfonamides and the fact that homosulfanilamide inhibited the growth of this strain—at very nearly the same concentration that

TABLE II

Inhibition of Group A Haemolytic Streptococci

Strain: C203. Medium: BPD. Inoculum: About 200 organisms. Read: At 24 hours.

DRUG	NO. OF TESTS	MINIMAL INHIBITORY CONCENTRATION		ACTIVITY RATIO (S/DRUG)
		Mean	Range	
		mgm. %	mgm. %	
D*	2	80	80, 80	1.6
Pz	2	20	<20, 20	6.5
Mrz	2	40	40, 40	3.2
Mtz	2	60	40, 80	2.1
Igm	2	130	160, 100	1.0
Igf	2	20	<20, 20	6.5
Homo	5	6	4–8	21.0
S	5	130	60–160	

* D = sulfadiazine, Pz = sulfapyrazine, Mrz = sulfamerazine, Mtz = sulfamethazine Igm = dimethylacroyl sulfanilamide (Irgamide), Igf = dimethylbenzoyl sulfanilamide (Irgafen), Homo = homosulfanilamide, S = sulfanilamide.

TABLE III

Inhibition of Group D Haemolytic Streptococci

Strain: Zymogenes. Medium: PDW. Inoculum: About 30 organisms. Read: After 1 day and 5 days.

DRUG	NO. OF TESTS	MINIMAL INHIBITORY CONCENTRATION		ACTIVITY RATIO
		24 hrs.	5 days	
		mgm. %	mgm. %	
D	1	>500*		2?
Mtz	1	>500		2?
S	2	>1000		
Homo	2	10	100	100

* See "Experimental Data—Drugs" for limits of solubility in broth.

sufficed for the Group A strain—clearly distinguishes this drug from the rest.

Homosulfanilamide also proved to be the most effective agent against five strains of staphylococci, Table IV. Irgafen, sulfamethazine, sulfa-

TABLE IV

Inhibition of Staphylococci—5 strains

Medium: PDW. Inoculum: 15 to 100 organisms. Read: After 1 day and 5 days.

DRUG	NO. OF TESTS	MINIMAL INHIBITORY CONCENTRATION			ACTIVITY RATIO
		24 hrs.		5 days	
		Mean	Range	Mean	
a) Staphylococcus aureus (Zorn), haemolytic, coagulase negative					
		mgm. %	mgm. %	mgm. %	
D	3	>500*	all > 500	>500	1?
Pz	3	>300	250 to >300	>300	2?
Mrz	3	>300	250 to >300	>300	2?
Mtz	3	250	200 to 300	650	2.8
Igm	3	500	400 to 600	>700	1.3
Igf	2	200	200, 200	400	3.4
Homo	3	37.2	<37.2 to 50	186	18.5
S	1	688			
b) Staphylococcus aureus, haemolytic, coagulase positive					
D	2	>500		>500	
Mtz	2	336		560	
Igm	2	>510		>510	
Homo	2	<37.5		75	
c) Staphylococcus aureus, haemolytic, coagulase positive					
D	2	>500		>500	
Mtz	2	445		>560	
Igm	2	>510		>510	
Homo	2	75		150	
d) Staphylococcus aureus, non haemolytic, coagulase positive					
D	2	>500		>500	
Mtz	2	336		560	
Igm	2	>510		>510	
Homo	2	<37.5		112	
e) Staphylococcus albus, non haemolytic, coagulase negative					
D	2	>500		>500	
Mtz	2	560		>560	
Igm	2	>510		>510	
Homo	2	75		186	

* See "Experimental Data—Drugs" for limits of solubility in broth.

TABLE V

Inhibition of Pneumococci

Strain: Type I, SV1. Medium: BPD. Inoculum: About 200 organisms. Read: At 24 hours.

DRUG	NO. OF TESTS	MINIMAL INHIBITORY CONCENTRATION		ACTIVITY RATIO
		Mean	Range	
		mgm. %	*mgm. %*	
D	2	8.0	8, 8	2.5
Pz	2	1.5	2, 1	13.3
Mrz	2	6.0	8, 4	3.3
Mtz	2	4.0	4, 4	5.0
Igm	2	8.0	12, 4	2.5
Igf	2	1.5	2, 1	13.3
Homo	5	6.0	4–10	3.3
S	5	20.0		

TABLE VI

Inhibition of E. coli

Strain: E. coli communis—old stock strain. Inoculum: 0.1 cc. of a 1:10,000 dilution (5000 to 25,000 bacilli).

DRUG	NO. OF TESTS	MINIMAL INHIBITORY CONCENTRATION *mgm. %*				ACTIVITY RATIO
		24 hrs.		5 days		
		Mean	Range	Mean	Range	
a) Medium: PDW						
D	1	150		>250		2.3
Pz	1	100		>250		3.4
Mrz	1	150		>250		2.3
Mtz	1	200		>250		1.7
Igm	1	150		>250		2.3
Homo	3	372	150 to 372	750	744 to >744	0.9
S	2	344		344		
b) Medium: S1G1						
D	2	0.1	0.05 & 0.1	0.5		20
Pz	2	0.05		0.5		40
Mrz	2	0.05		0.5		40
Mtz	2	0.50		0.75	0.5 & 1.0	4
Igm	2	0.50		5.0		4
Igf	2	1.5	1.0 & 2.5	5.0		1.3
Homo	8	186.0	93 to 186	400.0	186 to 465	0.01
S	5	2.0	<2 to 2.5	8.6	4.3 to 8.6	

merazine and sulfapyrazine were about equally effective while Irgamide, sulfadiazine and sulfanilamide were least bacteriostatic.

TABLE VII

Antagonistic Action of p-Aminobenzoic Acid

Strain: E. coli. Medium: S1G1. Inoculum: 0.1 cc. of a 1:10,000 dilution.

DRUG CONC. (MOLAR)	MINIMAL NEUTRALIZING CONCENTRATION OF PABA (MOLAR)				MEAN RATIO PABA/DRUG	
	24 hrs.		5 days			
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	24 hrs.	5 days
D 4×10^{-4}	6×10^{-5}	2×10^{-5}	6×10^{-5}	6×10^{-5}	1/10	1/66.6
Pz 4×10^{-4}	6×10^{-5}	2×10^{-5}	6×10^{-5}	6×10^{-5}	1/10	1/66.6
Mrz 4×10^{-4}	6×10^{-5}	2×10^{-5}	6×10^{-5}	6×10^{-5}	1/10	1/66.6
Mtz 4×10^{-4}	6×10^{-5}	2×10^{-5}	6×10^{-5}	2×10^{-5}	1/10	1/100
Igm 4×10^{-4}	6×10^{-5}	2×10^{-5}	6×10^{-5}	2×10^{-5}	1/30.8	1/100
Igf 4×10^{-4}	6×10^{-5}	2×10^{-5}	6×10^{-5}	2×10^{-5}	1/10	1/100

TABLE VIII

Inhibition of E. coli in the Presence and in the Absence of p-Aminobenzoic Acid

Medium: S1G1. Inoculum: 800–2200 organisms per cc. Read: After 1 day and 5 days; 3 tests without PABA, 2 with PABA.

DRUG	MINIMAL INHIBITORY CONCENTRATION (MGM. % × 100*)			
	Mean at 24 hrs.		Mean at 5 days	
	No PABA	1 mgm. % PABA	No PABA	1 mgm. % PABA
D	2^3	2^{13}	2^5	$>2^{13}$
Pz	2^3	2^{13}	2^5	$>2^{13}$
Mrz	2^3	2^{13}	2^5	$>2^{13}$
Mtz	2^5	2^{15}	2^7	2^{15}
Igm	2^6	$>2^{16}$	2^8	$>2^{16}$
Igf	2^7	$>2^{16}$	2^9	$>2^{16}$

* E.g. 2^3 means that a drug concentration of .08 mgm/100 cc. prevented growth. 2^{13} means that 81.92 mgm/100 cc. prevented growth.

With the Type I pneumococcus, as shown in Table V, homosulfanilamide assumed a middle position, while sulfapyrazine and Irgafen revealed the greatest activity and sulfanilamide the least. Finally, it is seen in Table VI that homosulfanilamide is the least active of all the compounds against a strain of E. coli. This difference is

emphasized in the simple S1G1 medium where the effect of the true sulfonamides is unhampered by antagonistic substances.

In Tables VII and VIII are shown the effects of p-aminobenzoic acid upon the bacteriostatic action of six of the compounds. Homosulfanilamide was not included in these experiments because, as mentioned above, it is not susceptible to this antagonist. When constant molar concentrations of the drugs were run against increasing molar concentrations of PABA (Table VII) very little difference was discernible between the compounds. In the second series of tests it was found (Table VIII) that higher concentrations of sulfamethazine, Irgamide and Irgafen were required to counteract 1 mgm. of PABA than was true with the other three drugs. At the same time, however, the ratio between the inhibitory concentrations with and without PABA appeared to be the same—2^{10} (10.24)—for all the drugs.

DISCUSSION

These results agree fairly well with the little that is known of the bacteriostatic properties of the several compounds. Thus, Schmidt and his associates (30) found sulfapyrazine to be more active than sulfadiazine against three strains of pneumococci. Högger (12) observed that Irgamide was inferior to sulfapyridine (which is usually ranked below sulfadiazine) in *in vitro* tests with pneumococci and haemolytic streptococci, although the two drugs were equally good *in vivo*. Marshall et al (29) report that against E. coli sulfadiazine has an "in vitro activity ratio" twice that of sulfamerazine (i.e., the former is twice as active as the latter) while we found no difference between the two. Hamre and her associates (31) found that homosulfamilamide was inhibitory for staphylococci at low concentrations.

In the present study sulfapyrazine proved to be the most active of the diazine derivatives against the Group A β haemolytic streptococcus, the pneumococcus and E. coli. It was inferior to sulfamethazine in tests with staphylococci, but we believe that this change in rank can be ascribed to the fact that sulfapyrazine is not soluble in broth, at pH 7.4, in concentrations much above 200 mgm. per 100 cc. Sulfadiazine was the least effective of this group of compounds in preventing the growth of the cocci. Against E. coli, however, it ranked with sulfamerazine and sulfapyrazine as is shown especially well in the tests in which PABA was used.

Irgamide appears to be only slightly more active than sulfanilamide *in vitro*, but Irgafen was the equal of sulfapyrazine in all tests except those with E. coli. Here there was such a marked descent in the scale of activity as to suggest for Irgafen a *specific* lack of effect for this organism. We are loath to accept such a suggestion. In this laboratory we have long held that the sulfonamide compounds were not specific for this or that organism. This belief was based on observations on sulfanilamide, sulfapyridine and sulfathiazole which invariably maintained the same order of activity, in the test tube, against the various bacteria. Lately, Marshall and his associates (29) have claimed a specific action on the part of certain of the sulfonamides in the control of experimental infections in mice. This, however, was not borne out *in vitro*, and there are several possible explanations for an apparent specificity *in vivo*. It is difficult, if not impossible, to conceive of specific behavior in the light of current theories of the mode of sulfonamide action. We therefore prefer to leave the question of Irgafen's seeming specificity in abeyance until further information has been obtained.

With homosulfanilamide this problem does not arise since the drug does not act through inhibition of the utilization of PABA—nor of its homologue, p-aminomethyl benzoic acid, according to Lawrence (25). Consequently it was not surprising to find that homosulfanilamide was outstandingly different from the other compounds included in these experiments. It was definitely more active than the others against both strains of haemolytic streptococci, and the staphylococci and definitely less active against E. coli. The difference was particularly striking in the tests with the Group D streptococcus and with E. coli grown in a synthetic medium. It is interesting that both Domagk and Miller and his associates found homosulfanilamide valueless against haemolytic streptococcal infections in mice. It seems probable that this lack of *in vivo* activity may be charged to faulty absorption or excretion of the compound, since Domagk also found that *local* applications cured gas gangrene in mice. This point has not been settled conclusively because of the difficulty of determining concentrations of this substance. Homosulfanilamide is not diazotizable. Using biological methods, however, we followed the urinary excretion of the drug in two patients and obtained evidence that it is excreted with great rapidity. We conclude from this that the concentration in

the blood must be low and inadequate for therapeutic purposes. Hence, we believe that the clinical value of homosulfanilamide will be limited to the local therapy of wounds and possibly the treatment of certain kinds of urinary tract infections.

SUMMARY AND CONCLUSIONS

1. The bacteriostatic properties of sulfadiazine, sulfapyrazine, sulfamerazine, sulfamethazine, N_1dimethylacroyl sulfanilamide (Irgamide), N_1dimethylbenzoyl sulfanilamide (Irgafen) and homosulfanilamide (Marfanil) have been tested against one strain each of Group A and Group D β-haemolytic streptococci, Type I pneumococci and E. coli, and five strains of staphylococci.

2. Sulfapyrazine was the most active of the diazine derivatives, but its effect was equalled by that of Irgafen except in tests involving E. coli.

3. Homosulfanilamide belongs in a different category. It was considerably more active than the other compounds against the two kinds of haemolytic streptococcus and the staphylococci and was less active against E. coli.

We wish to thank the following for the compounds used in this study:
American Cyanamid Company for sulfamerazine and sulfamethazine
Geigy Company, Inc. for dimethylacroyl sulfanilamide and dimethylbenzoyl sulfanilamide
Lederle Laboratories, Inc. for sulfadiazine and sulfanilamide
Mead Johnson & Company for sulfapyrazine
Squibb Institute for Medical Research for homosulfanilamide

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EXPERIMENTAL SHOCK[1]

II. Effects of Acute Plasmapheresis in Healthy Dogs

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In a previous paper (1) we reported the results observed when large quantities of whole blood are removed from healthy anesthetized dogs; in this paper we shall describe the effects of controlled removal of the fluid component of blood.

In traumatic shock abnormal amounts of extravascular serum-like fluid are regularly found in injured areas. It has been assumed that this fluid is derived from blood, that the volume of intravascular blood is reduced in consequence, and that such depletion is a factor—the main factor according to some investigators—in the production of the shock syndrome.

Furthermore, there is a widespread belief that severe shock, whatever the initiating cause, is associated with systemic losses of plasma through abnormally permeable capillaries.

If these assumptions are true, plasma loss must be viewed as an essential part of the mechanism of shock. Hence it is a matter of fundamental importance to know precisely the effects produced by controlled removals of plasma in the absence of such complicating factors as tissue injury, toxemia, and excessive or abnormal nervous stimuli. However, relatively few limited investigations of this problem have been reported.

Plasma may be removed from the circulation in measured amounts and at the rate desired by withdrawing whole blood, separating plasma from cells, and returning the cells to the vascular system. Those who

[1] Aided by a grant from the Smith, Kline and French Company, and by contributions from Mr. Alanson B. Houghton, Mr. Charles Moritz, and Mrs. Pierre Lorillard.

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introduced the method (2, 3, 4, 5) separated plasma and cells by centrifugalization and re-injected the cells suspended in physiological salt solution. Such a procedure obviously does not reproduce conditions thought to obtain in untreated shock, wherein plasma loss is not replaced by extrinsic fluid. To meet that objection Johnson and Blalock (6), and Harkins and Harmon (7) employed repeated rapid bleedings (e.g. amounts of blood equivalent to one per cent of the body weight), in each instance separating off a portion of the fluid and returning the cells suspended in a reduced amount of the subject's own plasma. The fault in this method is that during each period of centrifugalization the animal is deprived of a quantity of red cells as well as plasma. Roome, Keith, and Phemister (8) avoided this last difficulty by injecting previously prepared blood cells from a donor at the same time that the subject was bled.

EXPERIMENTAL METHOD

In the present investigation 24 dogs were used weighing from 6.3 to 20.4 kg., the average being 13.8 kg. Five of the animals had been splenectomized two or more weeks previously. The dogs were unselected as regards age, sex, and breed, but all were healthy, vigorous animals as shown by repeated preliminary measurements of temperature, hematocrit, plasma proteins, leukocytes, and blood pressure.

All the experiments were conducted uniformly under nembutal anesthesia. Our method of administering the drug and its observed physiological effects have been described (1). Morphine was not used.

After anesthesia each dog was observed for a time before institution of plasmapheresis. This period of quiet narcosis enabled physiologic functions to reach basal levels under standard conditions. Meanwhile many observations were made to determine the subject's normal or basal values for respiration, circulation, blood concentration, plasma volume, blood chemistry, oxygenation, etc.

For a full account of our experimental method and a critique of the procedures used the reader is referred to our initial publication (1). A few of the technics described there have been modified, however, in the interest of greater accuracy. A purified colorless preparation of heparin, generously supplied by Roche-Organon Company, was used latterly in the place of ammonium and potassium oxalate as an anticoagulant in preparation of plasma samples for falling-drop protein determinations. In calculating circulating blood volume and circulating cell volume, the amount of plasma trapped in the column of cells in hematocrit tubes was considered to be 8.5 per cent, as recommended by Chaplin and Ross (9), instead of five per cent as heretofore. Our method of measuring plasma volume has been described in detail elsewhere (10). The accurate measurement of plasma volume is a matter of supreme importance in a study of this sort; consequently, a great deal of care and

attention was devoted to it. In plasmapheresis it is necessary to take into account the amounts of dye removed by bleeding and the small amounts reinjected with cells.

Plasmapheresis was effected by removing whole blood in measured amounts, simultaneously injecting a volume of blood cells equal to the volume of cells removed by bleeding. At the beginning of each experiment, cells for replacement were obtained from a donor dog, but as the experiment progressed, the subject's own cells were used.

Specifically, blood was drawn from an artery in 40 cc. amounts with a 50 cc. syringe which contained 10 cc. of isotonic citrate solution. The resultant 50 cc. of citrated blood was transferred immediately to a 50 cc. centrifuge tube. When several such tubefuls had been collected, they were spun horizontally for 25 minutes at maximum speed in an International Type 2 centrifuge. After the supernatant plasma had been aspirated off as carefully and completely as possible, the residual cells, including platelets and leukocytes, were pooled and reinfused without addition of salt solution or other fluid. Although the hematocrit readings of these highly concentrated suspensions of cells ranged from 95 to 99 per cent, the viscous material was introduced readily through a burette attached to a cannula in a vein. Throughout the procedure precautions were taken to avoid hemolysis and minimize sepsis. An effort was made also to keep close track of the changing cell-plasma ratio in the circulation. Because of a necessary lag of 45 minutes or more in measuring the hematocrit, replacement of cells, volume for volume, could be only approximate. Final calculations at the end of experiments usually showed that we had missed exact replacement by small amounts.

Account was kept also of the small quantities of citrate and plasma injected with cells, since these additions to the circulation enter into calculations of plasma volume and fluid shifts.

Plasmapheresis was continued until the animal was thought to be in a state of shock from which he would not recover spontaneously. That condition could not always be recognized with certainty, however. Blood pressure was found to be a less reliable criterion in plasma-loss shock than in post-hemorrhagic shock. Hemoconcentration, excessive respiratory exchange, and reduction in oxygen uptake were considered useful signs of developing shock, though no constant "shock level" was observed for any of these conditions. It sometimes happened that an animal in an indeterminate state of shock would be precipitated into a rapidly fatal condition by the removal of a few additional cc. of plasma.

Technically plasmapheresis is much more difficult to carry out in a properly controlled manner than is hemorrhage. The procedure tends to be long drawn-out, for as the blood becomes more concentrated, successive exchanges of the cells for whole blood remove less and less plasma from the circulation.

As heretofore, we shall express our results mainly in the form of averages (arithmetical means), but follow them parenthetically where it seems indicated with certain statistical data—number of observations (n), range of variation (R), and standard deviation (σ).

EFFECTS OF ACUTE PLASMA LOSS UPON DOGS

Twenty-one dogs were subjected to plasmapheresis until signs of shock developed. Eighteen of the animals died; the remainder would certainly have died had successful therapy not been instituted. The amounts of plasma removed averaged 26.3 cc. per kg. of body weight. Three dogs with sublethal plasmapheresis recovered spontaneously. Average duration of the basal or control period (from introduction of anesthesia to beginning of plasmapheresis) 3 hours, 33 minutes; average duration of the period of plasmapheresis 4 hours, 26 minutes; average time from beginning of plasmapheresis to death 5 hours, 53 minutes.

Effects of acute plasmapheresis upon the dynamic aspects of the circulation: A progressive fall in mean arterial pressure was noted. In individual experiments blood pressure tracings were less smooth than the curve shown in figure 1 due to fluctuations caused by intermittent removals of plasma. Mean arterial pressure at the beginning of plasmapheresis averaged 123 mm. of Hg (n 24, R 163–86 mm., σ 15.5 mm.); at the end of plasmapheresis 77 mm. (n 24, R 131–36 mm., σ 20.7 mm.); at the sudden "break" 54.5 mm. (n 21, R 76–35 mm., σ 12.2 mm.). Analagous averages in our hemorrhage experiments (1) are shown in Table 5.

The cardiac output curve declined more rapidly than the blood pressure curve (figure 1). A like result was noted after hemorrhage, but the discrepancy is more pronounced in plasmapheresis, due, presumably, to the viscosity of blood in plasmapheresis and resultant high peripheral resistance. Average cardiac output in this series of experiments in cc. per minute per sq. m. of body surface was as follows: Basal 3.49 (n 13, R 1.91–5.55, σ 1.01), at the end of plasmapheresis 1.09 (n 11, R 0.47–3.43, σ 0.81), at the "break" 0.90 (n 10, R 0.44–3.43, σ 0.85).

Venous pressure fell more rapidly than arterial. By the end of plasmapheresis the peripheral veins at heart level usually appeared empty.

Reduced volume flow of blood through the extremities was an early and pronounced change. Even in late stages of plasma-loss shock, however, a slight irregular flow of blood through an extremity could be detected by means of a plethysmograph.

Effects of acute plasmapheresis upon blood volume and body fluids: Plasma volume was measured by means of the dye T-1824 using our modification of the Gregersen-Gibson method (10). Samples of blood for this purpose were taken every hour or oftener throughout the experiments. In the present series of experiments initial plasma volumes averaged 51.1 cc. per kg. of body weight (n 24, R 36.3–72.5 cc., σ 9.12

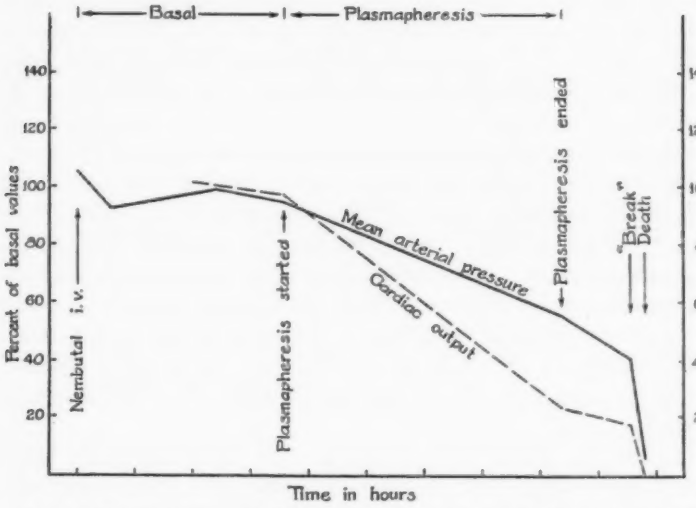

FIG. 1. Composite curves[4] showing the effects of acute plasmapheresis upon mean arterial blood pressure and cardiac output.

cc.). The initial volume was always reduced slightly during the control period and very greatly, of course, during the period of plasma removal. Fluid shifts into and out of the blood vascular system are indicated in figure 2 and Table 1. As was true after hemorrhage,

[4] Because the experiments varied considerably in duration, figures 1–5 are necessarily semi-diagramatic. The curves are drawn from averages of analagous readings of all experiments on a time-scale that is itself a mean. In individual experiments, blood pressures were recorded on a kymograph continuously, and other curves were plotted from hourly or half-hourly readings. To facilitate comparison, most of the curves are drawn on a percentage scale, basal values being 100.

shock following plasmapheresis was attended by relatively little change in circulating plasma volume even during the final stages of circulatory

TABLE 1

Effects of Plasmapheresis upon Plasma Volume and Fluid Shift

Average for 21 dogs. Plasma removed in blood sampling included in the totals.

	PLASMA REMOVED DURING EXPERIMENT	OBSERVED REDUCTION IN TOTAL PLASMA	NET FLUID SHIFT INTO (+) OR OUT OF (−) THE PLASMA
	cc./kg.	*cc./kg.*	*cc./kg.*
By the beginning of plasmapheresis	1.5	2.7	−1.2
By the end of plasmapheresis	25.8	22.7	+3.1
By the time of the break in B.P.	26.2	25.2	+1.0
By the time of death	26.3	25.3	+1.0

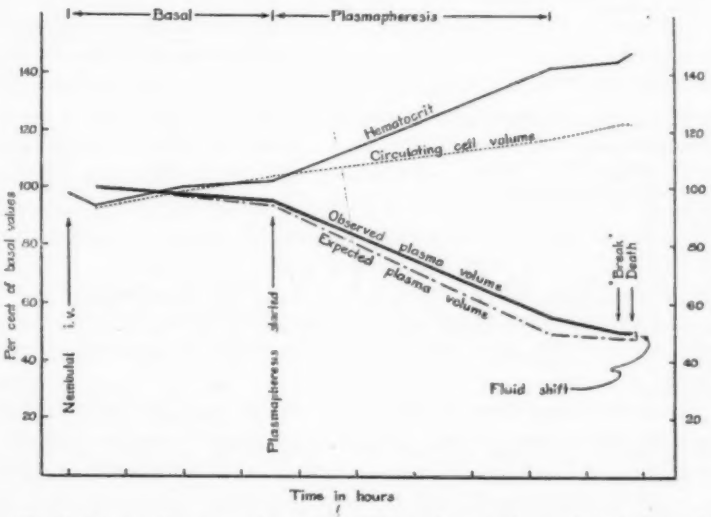

FIG. 2. Composite curves showing the effects of acute plasmapheresis upon the volume and concentration of circulating blood cells, and the volume of plasma.

collapse. This observation has a bearing on the problem of capillary permeability in acute plasmapheresis shock.

The total volume of actively circulating blood cells, estimated by the formula

$$\text{C.C.V.} = \frac{\text{P. V.}}{100 - (\text{Ht.} \times 0.915)} - \text{P. V.}$$

(C.C.V. = circulating cell volume; P. V. = plasma volume; Ht. = hematocrit) showed the usual variations during the control period. In all probability there was an initial decrease in volume as a result of inactivity and nembutal; this was followed by a moderate rise thought to be due to late nembutal reaction, cannulating, blood sampling, etc. During and after plasmapheresis, however, there occurred a large increase in the absolute amount of circulating blood cells (figure 2 and

TABLE 2

Effects of Plasmapheresis upon "Circulating Blood Cell Volume"

	OBSERVED CIRCULATING CELL VOLUME	EXPECTED CIRCULATING CELL VOLUME	CELL SHIFT (INTO THE BLOOD STREAM)
	cc./kg.	*cc./kg.*	*cc./kg.*
At the beginning of experiment	36.4	36.4	0
At beginning of plasmapheresis	42.4	35.1	+7.3
At the end of plasmapheresis	46.9	33.2	+13.7
At break in blood pressure	49.2	32.5	+16.7
At death	48.9	32.2	+16.7

Table 2). This was a constant finding. Even the splenectomized dogs showed similar, though smaller, increases in the total circulating cell volume. No such increase was observed in post-hemorrhagic shock.

Since no change could be detected in the average size of individual red cells, the increase in total circulating cell volume must be attributed to activation of stagnant or stored blood cells. Furthermore, in order to produce the observed rise in cell-plasma ratio these cells must be added to the circulation in concentrated form; that is, suspended in a relatively small amount of plasma.

In view of the fact that plasma withdrawal of itself raises the hematocrit and increases the viscosity of circulating blood to a dangerous degree, it is surprising that the situation should be worsened by having

additional cells thrown into active circulation. Reviewing these experiments in detail, one is led to believe that this strange reaction contributed materially to decompensation and death in our animals.

No significant changes could be detected by the thiocyanate method[5] in the quantity of "available" body fluids following plasmapheresis.

Plasmapheresis produced first oliguria, then anuria. Mean arterial pressures regularly associated with these effects were: Definite slowing of output at about 100 mm. Hg; cessation of output at 60–70 mm; reappearance of normal urine at approximately 80 mm.

Insensible loss of water, which averages about 0.6 cc. per kg. per hour under basal conditions, was nearly tripled by plasmapheresis. This increase was attributed to hyperpnea.

Effects of acute plasmapheresis upon concentration and morphology of circulating blood cells: Rest alone reduces hematocrit readings of active, non-splenectomized dogs by six or eight points; nembutal causes a further reduction and there is an associated increase in size of the spleen. The postanesthesia fall in hematocrit in our present series of experiments was followed by a slow rise (figure 2) due in part perhaps to the stimuli of minor operative procedure and blood sampling. Plasmapheresis produced great changes in the cell-plasma ratio. Average hematocrit readings: After induction of anesthesia 45.0 (n 24, R 34.8–63.6, σ 3.5); before plasmapheresis 50.9 (n 24, R 37.4–69.9, σ 7.7); after plasmapheresis 68.4 (n 23, R 46.6–82.4, σ 7.4); at the "break" 70.7 (n 20, R 52.9–84.6, σ 6.6); and at death 72.1 (n 9, R 63.2–85.5, σ 7.5). The rise in hematocrit was due partly to experimental depletion of plasma and partly to the aforementioned absolute increase in the volume of circulating cells. In these animals there was relatively little shift of fluid from the tissues to the blood (Table 1) to counteract the red cell concentration.

Almost identical curves were obtained for hematocrit and hemoglobin; and red cell counts made in a few experiments showed roughly similar variations. It may be said, therefore, that acute plasmapheresis does not alter appreciably the average size or hemoglobin content of red cells. Moreover, blood cells from the shocked animals showed no significant changes in size, shape or color of erythrocytes. Ghost

[5] The limitations of this test should be recognized, however. A critique of the method was presented in our first publication (1).

and stippled cells were rarely seen, and in the majority of cases colorimetric examination failed to reveal hemolysis in samples of plasma.

Whereas in post-hemorrhagic shock close agreement was observed between the curve of arterial oxygen content and the curves of hematocrit and hemoglobin, in plasmapheresis (12 experiments) the increase in oxygen content consistently failed to keep pace with the increase in hematocrit and hemoglobin (figure 4). Moreover, these gasometric tests were made on samples of blood drawn for cardiac output examinations while the animals were breathing pure oxygen. It is possible that the incomplete oxygenation of venous blood during its passage through the lungs was even more pronounced while the animals were breathing room air.

Inconstant degrees of leukocytosis were observed following plasmapheresis. Since other factors were present in all of these experiments it is not possible to say what effect plasma loss alone had upon the white cell count.

Effects of acute plasmapheresis upon plasma proteins and nitrogen metabolism: Plasma protein concentration, which averaged 6.33 grams per cent (n 23, R 5.34–7.06 grams, σ 0.77 grams) at the outset, usually remained unchanged during the control period, but was reduced slightly during plasmapheresis by dilution with water from the tissues (figure 3). The absolute amount of plasma protein (plasma volume x protein concentration) was reduced by removal of plasma, but thereafter no significant changes could be detected (Table 3 and figure 3). That there was no unaccountable reduction in the intravascular plasma protein even as death approached is further indication of the absence of abnormal capillary permeability to proteins in this particular type of shock.

Following plasmapheresis the non-protein nitrogen of circulating plasma rose. The average result in six experiments is shown in figure 3.

Lymph was collected from the thoracic duct in only one experiment. No significant changes in its protein concentration followed plasmapheresis.

As has been mentioned, plasma depletion is associated with a reduced output of urine. There was also a great reduction in the excretion of urinary nitrogen. What urine was excreted had a high specific gravity, but albuminuria was rarely observed.

In general, it may be said, therefore, that acute plasmapheresis produced relatively minor disturbances of ordinary nitrogen metabolism,

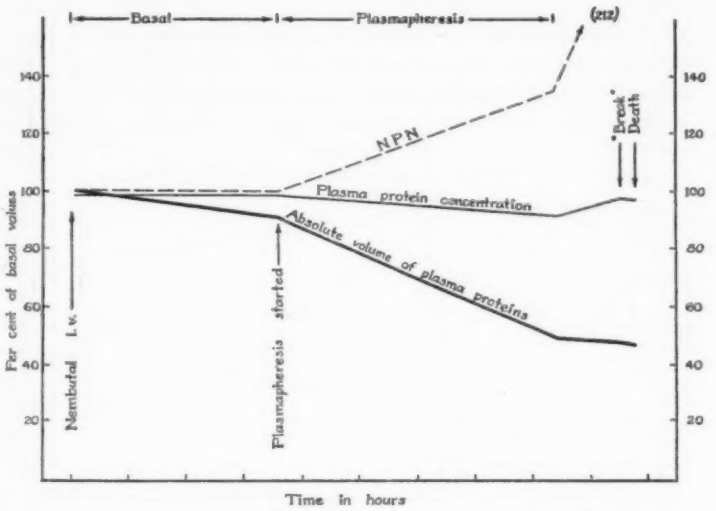

FIG. 3. Composite curves showing the effects of acute plasmapheresis upon plasma proteins and b ood non-protein nitrogen.

TABLE 3

Effects of Acute Plasmapheresis upon Total Intravascular Plasma Proteins

Averages for 14 dogs

	OBSERVED TOTAL PLASMA PROTEINS	EXPECTED TOTAL PLASMA PROTEINS	DIFFERENCE
	gm./kg. body wt.	*gm./kg. body wt.*	*gm./kg. body wt.*
At beginning of experiment	3.27	3.27	0
At start of plasmapheresis	2.95	3.04	−0.09
At end of plasmapheresis	1.72	1.82	−0.10
At break in blood pressure	1.58	1.56	+0.02

and that those disturbances played little or no part in bringing about circulatory collapse and death in our animals.

Effects of acute plasmapheresis upon respiration: Plasmapheresis increased both the rate of respiration and the minute volume exchange

of air (figure 5). This excessive respiratory exchange did not result in full oxygenation of the highly concentrated pulmonary blood, however. The hyperventilation facilitated elimination of carbon dioxide

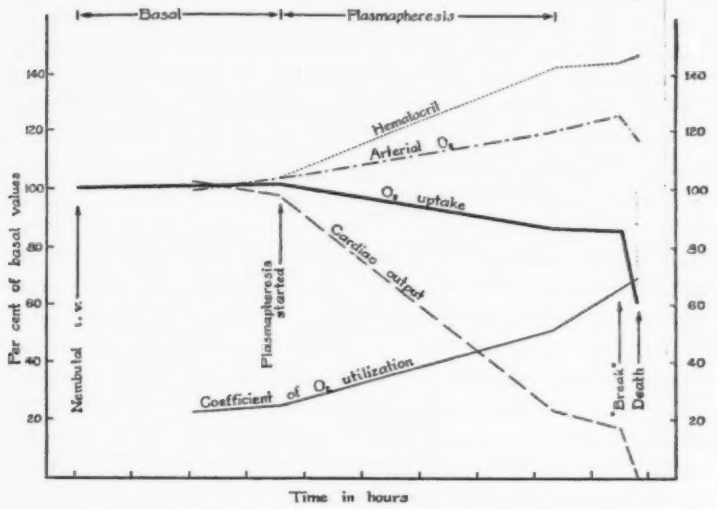

FIG. 4. Composite curves showing the effects of acute plasmapheresis upon oxygenation.

TABLE 4

Effects of Acute Plasmapheresis Upon O_2 and CO_2 of Blood

(Average of 16 Experiments)

	ARTERIAL O_2	VENOUS O_2	VENOUS CO_2	ARTERIAL CO_2
Before plasmapheresis	21.36	16.07	36.06	31.41
After plasmapheresis	25.56	10.29	29.09	14.30
Near break in B.P.	25.59	6.98	28.46	12.38

from the lungs and reduced its concentration in blood (Table 4). Concomitantly the alkali reserve in the blood was reduced (figure 5).

Oxygen uptake in this series of dogs under basal conditions of nembutal anesthesia averaged 7.34 cc. per minute per kg. of body weight.

In normal animals need, uptake, and use of oxygen are all equal and are kept nicely balanced by complex automatic physiologic adjustments, but in shocked animals oxygen need may exceed oxygen supply. That discrepancy is the true measure of anoxia in the body as a whole. The fall in oxygen uptake shown in figure 4 represents a reduction in oxygen supply to the body, although there is no reason to believe that these animals after plasmapheresis needed less oxygen than they did under basal conditions. It is far more likely that oxygen need actually

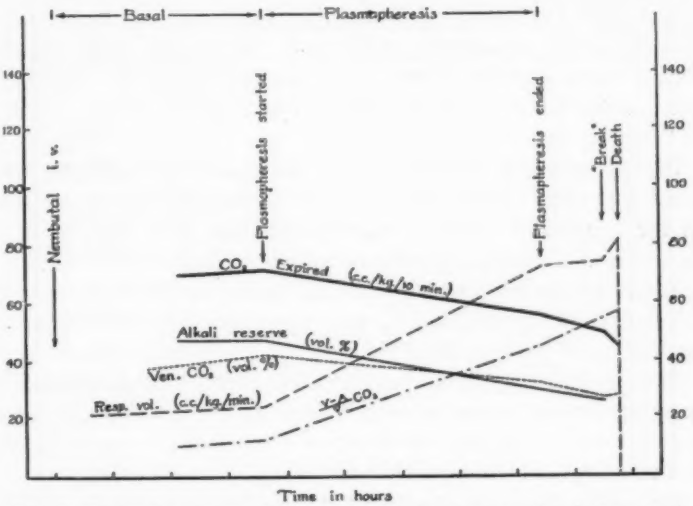

FIG. 5. Composite curves showing the effects of acute plasmapheresis upon respiration and CO_2 elimination.

increased because of the slight rise in body temperature and the greatly increased activity of muscles of respiration. Falling alkali reserve (figure 5) is further evidence of imcomplete oxygenation. It may be assumed, therefore, that anoxia was present in these animals, that it increased with the development of shock, and that oxygen lack finally became acute.

The minute volume elimination of carbon dioxide by the lungs was reduced in these experiments (figure 5). Respiratory quotients were as follows: Before plasmapheresis 0.88; after plasmapheresis 0.93; at

or near the break in blood pressure 0.86. The proportion of venous carbon dioxide exhaled

$$\frac{V_{CO_2} - A_{CO_2}}{V_{CO_2}},$$

which may be termed the coefficient of elimination of CO_2, rose as the experiments progressed.

Other effects of acute plasmapheresis: Narcosis was prolonged and was deepened somewhat by severe plasma loss.

The effect of plasmapheresis upon the heart rate was variable, but most of the dogs manifested an increasing tachycardia. Whereas following hemorrhage death was almost always respiratory, in plasmapheresis death was sometimes observed to follow an abrupt cessation of the heart beat.

Plasma potassium measured in only two experiments showed a sharp terminal increase. Averages for the two experiments in mgs. per cent: At the outset 29.5, before plasmapheresis 26.1, after plasmapheresis 28.2, at the break in blood pressure 38.8.

Temperature changes (average of all cases): At the outset 99.5 F., before plasmapheresis 101.4 F., after plasmapheresis 101.8 F., at the break in blood pressure 101.5 F.

One dog out of 24 passed bloody stools during the experiment. This gross bleeding can scarcely be attributed to plasmapheresis, however, since the same thing has been observed occasionally in unshocked animals during the control period under nembutal anesthesia.

No significant change was observed in the clotting time of blood following plasmapheresis.

Postmortem examination: The moment of death was not associated with any appreciable anatomical changes which might help to explain sudden respiratory and circulatory failure which terminated the experiments. The abdomen of one dog was opened widely just before death, but no gross alterations could be observed at or immediately after the death of the animal; there was no rapid vasodilation, increase of congestion, venous engorgement, change in size of spleen, or variation in wetness of tissues.

All of the animals showed a fairly uniform picture. There was slight congestion of all tissues in striking contrast to the uniform pallor

observed after massive hemorrhage. The large veins were moderately well filled. Spleen, kidneys, and liver contained dark, viscous blood, but were not distended. The tissues everywhere appeared normally moist. Serous cavities contained the usual small amounts of fluid. Hemorrhages were observed occasionally in the spleen. About one-fourth of the cases had minute hemorrhages in the mucosa of the small intestine. Only rarely was any blood-stained material found in the intestinal lumen. Hemorrhages in the adrenal, so common in shock due to severe blood loss, were found only occasionally in animals dying from plasmapheresis shock. The lungs, heart, and the central nervous system appeared normal, both grossly and microscopically.

DISCUSSION

A controversial question is whether or not shock produced by hemorrhage alone is similar to the shock produced mainly by local fluid or plasma loss. The problem has often been confused by attempts to compare the effects of hemorrhage and of plasma loss in the presence of other variables such as pain, toxemia, chemical irritation, nervous stimuli, severe emotional strain, and anesthesia. In our two series of experiments plasma and whole blood were removed under almost identical conditions; hence comparison seems valid. Some of the more important similarities and differences are listed in Tables 5 and 6.

It is clear from such a comparison that the two conditions show similarities, but that they also differ in many respects. After blood loss there is usually slight hemodilution, and after plasma loss there is a high degree of hemoconcentration; although in both conditions systemic anoxia finally occurs. In hemorrhage, mean arterial blood pressure is a fairly reliable index of shock but the hematocrit is unreliable. In acute plasmapheresis, on the other hand, the hematocrit is often the more dependable criterion. One gets the impression that vasoconstriction is a less prominent factor in plasmapheresis than it is in hemorrhage: cardiac output is much the same in plasmapheresis as in hemorrhage even though the blood is much more viscous; blood flow in the extremities does not cease entirely for all its viscosity; and hemorrhages in the adrenals are not so pronounced after plasmapher-

TABLE 5

Comparison of Acute Hemorrhage and Acute Plasmapheresis—Averages of All Experiments

AMOUNTS OF CELLS AND PLASMA REMOVED TO PRODUCE SHOCK (IN CC./KG. BODY WEIGHT)	HEMORRHAGE	PLASMAPHERESIS
Cells	15.5	3.41
Plasma	20.3	26.28
Total	35.8	30.69
Survival time after beginning of shock production	5° 22′	5° 53′
Mean arterial pressure (in mm. Hg)		
At beginning of shock production	126	123
At end of shock production	63	77
At time of break in blood pressure	47	54.5
Cardiac output (in cc./sq.m./min.)		
At beginning of shock production	3.51	3.49
At end of shock production	1.05	1.09
At time of break in blood pressure	0.91	0.90
Hematocrit—basal	44.7	45.0
At end of shock production	45.6	68.4
At break in blood pressure	44.8	70.7
At death	44.1	72.1
Maximum shift of blood cells from stores to active circulation (in cc./kg. body weight)	4.7	16.7
Measured plasma volume (in cc./kg. body weight)		
At beginning of shock period	49.0	48.4
At end of shock period	33.7	28.4
At break in blood pressure	33.5	25.9
Per cent reduction in original plasma volume	to 66.7%	to 50.7%
Ultimate shift of water from tissues to plasma (in cc./kg. body weight)	3.8	1.0
Estimated volume of circulating blood (plasma volume plus circulating cell volume) (in cc./kg. body weight)		
At beginning of shock period	93.1	90.8
At end of shock period	62.0	75.3
At break in blood pressure	60.7	75.1
Increase in respiratory exchange	x 5.1	x 4.0

esis as they are after massive hemorrhage. Uncomplicated posthemorrhagic death follows a fairly uniform pattern: The blood pressure declines slowly, breaks sharply, and death occurs ten to twenty minutes later; during the last two or three minutes the heart rate changes from rapid to slow, and respirations, which had been deep and fast, suddenly falter before the heart stops beating. The course of acute plasmapheresis may be similar; but in many cases, especially when the hematocrit is very high, the heart stops suddenly, blood pressure, which

TABLE 6

Difference between Effects of Acute Hemorrhage and Acute Plasmapheresis

	ACUTE HEMORRHAGE	ACUTE PLASMAPHERESIS
Blood	Slightly diluted	Extremely concentrated and viscous
Fluid shift from tissues to blood	Moderate	Slight
Activation of red cell stores	Moderate to absent	Very great
Criteria of shock	Blood pressure fairly dependable; hematocrit of no value	Blood pressure not dependable; hematocrit very helpful
Reduction in cardiac output	Roughly proportional to blood pressure	Out of proportion to blood pressure
Pulmonary blood	Completely oxygenated (about 95% saturated)	Incompletely oxygenated
Death	Always respiratory	Sometimes respiratory; at other times cardiac
Tissues	Pale	Slightly congested
Hemorrhage in adrenals	Almost always present	Often absent
Venous trunks	Less than normally full	Moderately well filled

may have been comfortably high, plummets to zero, and the animal, after a few spasmodic respiratory efforts, dies. Finally, the postmortem appeance of organs and tissues is somewhat different in the two conditions, although in neither case is there much evidence of "pooling" of blood, extensive pectechial hemorrhages, or systemic capillary permeability.

It should be pointed out that in hemorrhage a shift of blood cells from storage into active circulation compensates in part for the cells removed by bleeding. The experiment, therefore, is really one of less-

than-stated blood loss plus some plasma loss. In plasmapheresis, on the other hand, the blood cell shift into the active circulation is so great that it far more than compensates for any cells removed by blood sampling or lost in minor operative procedures. Consequently the effects are purely those of plasma loss.

In acute plasmapheresis the shift of red cells is large—in some instances almost incredibly large. These measurements were carefully and critically made, however, and we have no reason to doubt their essential correctness. Much smaller shifts occurred in the five splenectomized animals.

In view of the generally held opinion that anoxia causes abnormal capillary permeability, it should be noted that the present series of experiments does not support that thesis. Evidence that our shocked animals suffered from anoxia is provided by the reduced oxygen uptake and the lowered blood alkali reserve during shock. Reduced venous oxygen is indicative of a high coefficient of utilization of oxygen, not necessarily anoxia of the tissues; however, the very low oxygen content of the venous blood in the late stages of plasmapheresis shock certainly suggests the presence of anoxia. The absence of abnormal capillary permeability in these anoxic animals is evidenced by unchanged disappearance rates of T-1824 throughout the experiments. This dye, when injected intravenously, is promptly and firmly attached to plasma protein molecules. Consequently, losses of protein from the vascular system are reflected in proportionate losses of dye. Additional evidence of normal capillary permeability in our shocked dogs is provided by the absence of unaccountable reductions in plasma volume or in the absolute quantity of intravascular plasma protein (Tables 1 and 3). Furthermore, there was no sudden rise in hematocrit during the period of anoxia to indicate uncontrolled plasma loss. Finally, confirmatory evidence is provided by additional experiments to be reported separately in which massive intravenous injections of T-1824 show clearly that abnormal leakages of dye (and protein) from the vascular bed into the tissues do not occur following uncomplicated acute plasmapheresis.

The following is an attempt to interpret and integrate the changes observed after acute plasma loss, and to trace the course of events from plasmapheresis to circulatory collapse and death.

A. *Primary effects of acute plasma loss:* These are hemodynamic: reduced blood volume, increased blood viscosity, decreased blood flow through the heart and body tissues, and low arterial and venous pressures. Fundamentally they are simple physical effects which have been reproduced in a mechanical circulation model (11) by reducing the volume and increasing the viscosity of circulating fluid. In the animal, however, these physical effects are modified by physiologic adaptations which will be discussed under heading C.

B. *Secondary effects of acute plasma loss:* If not compensated for, the vital functions of blood are necessarily disturbed by reduction of its volume, by slowing of its rate of flow, and by incomplete pulmonary oxygenation. A function of blood is to supply adequate amounts of oxygen continuously to the tissues, particularly to vital centers and organs. Severe anoxemia quickly leads to dysfunction and death. Other vital processes such as elimination of CO_2, regulation of body temperature, water and electrolytic balances, nutrition, and hormonal activities are likewise dependent upon an efficient blood circulation. Consequently, depression of the circulation by plasmapheresis might be expected to produce a variety of functional and organic abnormalities. Actually few were found in these acute experiments. The reason, perhaps, is that oxygenation is the function of blood most promptly and seriously deranged by severe plasma loss, and animals succumb from anoxia before other pathologic changes have had time to become manifest. Furthermore, the paucity of abnormal anatomical changes, gross or microscopic, suggests that the deleterious effects of anoxia are largely functional.

The sudden failure of the circulation in late stages of plasmapheresis shock is not easy to explain satisfactorily. No abrupt vasodilatation, change of heart action, alteration in volume or concentration of blood, or abnormal capillary permeability was detected at the time of the "break" in blood pressure. As previously mentioned, some of our animals with high hematocrit and well sustained blood pressure collapsed rapidly and died without warning, as though the heart, exhausted by attempts to force viscous blood through the vascular system, suddenly gave out entirely. There was no evidence of acute dilatation of heart in those cass.

The secondary pathological effects of acute plasmapheresis, though

vaguely manifested, are of the utmost importance. They may be fatal. Anoxia resulting from an impaired circulation tends reciprocally to depress the circulation further, and this vicious circle, if not broken, may lead rapidly to collapse and death.

Results of therapy will be reported in a subsequent paper, but it may be noted in this connection that replacement therapy is somewhat more successful in saving the animals in late stages of blood loss shock than the animals in late stages of plasma loss shock. This observation raises the question of whether "irreversible changes" are not more apt to occur in the latter condition.

C. *Compensatory physiologic adjustments:* Small or moderate reductions of plasma volume do not usually produce the effects just described because a number of physiologic mechanisms are set in operation which compensate for such losses. It is only when the volume of plasma is seriously depleted that compensation fails and signs of shock develop.

(1) The heart promptly begins to beat more forcefully and rapidly, tending to maintain normal cardiac output and arterial blood pressure. (2) A degree of selective vasoconstriction occurs, shunting blood from non-vital regions such as the skin through the more vital centers. This compensatory mechanism appears to be less effective in plasmapheresis than in hemorrhage, because, as has been mentioned, vasoconstriction is probably less pronounced and because the blood is much more viscous. (3) A small shift of fluid from the tissues increases the volume of plasma and tends to lessen hemoconcentration; at the same time activation of red cell stores supplements the depleted blood volume, but at the cost of hemoconcentration. (4) The coefficient of utilization of oxygen is increased, so that hemoglobin gives up 60 to 70 per cent of its load of oxygen instead of the usual 15 to 20 per cent. This adjustment is perhaps the most important and effective of these compensatory phenomena. (5) Hyperventilation increases progressively. It facilitates oxygenation of pulmonary blood and aids in elimination of CO_2.

These compensatory mechanisms provide a wide margin of safety for normal individuals. That margin is narrowed by plasma loss. When the loss is large, compensation eventually fails and anoxia develops as illustrated by figures 4 and 5. Whenever that happens spontaneous recovery is improbable.

D. *Regeneration of plasma:* In untreated animals, after substantial but sublethal plasma losses, water begins at once to be added to the circulating blood; however, 24 to 48 hours may be required for full restoration of the plasma volume. Replacement of the lost plasma proteins likewise starts promptly, but progresses slowly. In one experiment in which we removed 26 per cent of the original plasma and subsequently permitted the dog to eat pellets and drink water *ad lib.*, the absolute quantity of plasma proteins returned to the original value almost exactly 48 hours after the onset of plasmapheresis. When lethal amounts of plasma have been removed, the relatively slow process of regeneration is interrupted in its early stages by death of the animal.

SUMMARY

Plasma loss is a factor of prime importance in many types of clinical shock. Such losses are known to occur locally in traumatized regions; many investigators believe that plasma is also lost systematically through abnormally permeable capillaries. In an effort to determine the precise effects produced by plasma loss under standard experimental conditions, and in the absence of trauma or other complicating factors, 24 dogs were subjected to acute plasmapheresis. The results of those experiments are reported in detail.

The observed changes are interpreted as follows: The primary consequence of acute plasma loss is a direct disturbance of hemodynamics. The resultant circulatory depression affects the body secondarily by interfering with functions of the blood, especially the function of oxygenation. Compensatory physiologic adjustments occur, but progressively less adequately, until decompensation develops with resultant anoxia of vital centers and organs. Regeneration of plasma occurs too slowly to save these acutely and severely shocked animals. Dysfunction or failure of the vital organs further depresses the circulation and this vicious circle leads finally to stoppage of circulation, irreparable organic damage, and death. Successful therapy interrupts and tends to reverse that course of events. In sublethal plasmapheresis decompensation may not occur at all, or decompensation of a relatively monor degree may develop, which is relieved in time by plasma regeneration.

Evidence is presented to show that abnormal capillary permea-

bility did not occur in the animals shocked by acute plasmapheresis, although anoxia was present.

A comparison is made of the effects of acute plasmapheresis and of acute hemorrhage under similar experimental conditions. The states of circulatory depression which result have some features in common, but they also differ in many respects, justifying the conclusion that the two procedures produce distinctive types of shock.

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TRYPTOPHANE DERIVATIVES IN THE URINE OF PYRIDOXINE-DEFICIENT SWINE[1]

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It has been known for some time that in rats and rabbits the metabolism of tryptophane proceeds through kynurenine to two quinoline derivatives, xanthurenic acid and kynurenic acid (1). Kynurenine was first isolated as an intermediate product of tryptophane metabolism from the urine of rabbits fed a diet of polished rice (2). This diet is known to be deficient in the vitamin B complex. Lepkovsky, Roboz and Haagen-Smit (3) have recently presented evidence of a relationship between tryptophane and pyridoxine metabolism. They have identified the green pigment-producing substance, which they found in the urine of pyridoxine-deficient rats, as xanthurenic acid.

The administration of either *d*- or *l*-tryptophane to rabbits results in kynurenine formation (4). However, *d*-kynurenine does not give rise to kynurenic acid (5). Berg (6) has shown that although both *d*- and *l*-tryptophane are equally effective in supporting growth in the rat, kynurenic acid is formed only from *l*-tryptophane.

It is the purpose of this paper to study the occurrence of these metabolic products of tryptophane metabolism in the urine of pyridoxine-deficient pigs, and also to report on the presence of an additional pigment.

METHODS

Full details of the experimental method have been published elsewhere (7). Pigs three weeks of age were obtained from the Bureau of Animal Industry, the United States Department of Agriculture, Beltsville, Maryland, and were fed synthetic diets. The control and pyridoxine-deficient diets have been described elsewhere (8). The pyrid-

[1] These studies were aided by grants from the Rockefeller Foundation, Park-Davis and Company, and the Upjohn Company, and were carried out in cooperation with the Bureau of Animal Industry of the U. S. Department of Agriculture.

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oxine-deficient low-protein basal diet consisted of casein, 10 per cent; sucrose 73.8 per cent; lard, 11 per cent; swine salt mixture (7), 5.2 per cent. Tryptophane (Merck & Co.) when given to the animals was administered orally, in capsules, in doses of 0.5 grams of *dl*-tryptophane each day. Urine collections were made under toluene for twenty-four hour periods, the urines were immediately acidified with hydrochloric acid so that they were acid to litmus and thereafter were stored in a refrigerator.

"Kynurenine" excretion in the urine was measured quantitatively by the method of Kotake and Kawase (9) with one alteration. An equivalent amount of sodium hydroxide was used in place of barium hydroxide, to keep the volume in the distilling flask at a minimum. Identical results were obtained with this modification. Kotake and Kawase report that their method is quantitative and specific, but it seems reasonable that there may be other substances in the urine which are precipitated by mercuric sulfate and which have labile amino groups. Since identification of kynurenine by isolation was not attempted, the term is used in this paper to refer to all substances precipitable by mercuric sulfate and containing labile amino groups. The results were calculated as kynurenine.

Xanthurenic acid excretion was measured qualitatively by neutralizing the urine to litmus, adding a few drops of ferric ammonium sulfate solution and filtering (3). So far as is known there are no substances which interfere with this test. The depth of the green color was recorded in plus values ranging from one to four. When the green color was just sufficient to be identifiable without question, the reading was one plus. When the color was so deep that it was almost black, the specimen was designated "four plus." Two and three plus were intermediate between these extremes.

Kynurenic acid was determined on twenty-four hour urine specimens by the Capaldi isolation method outlined by Berg (10). All crude Capaldi precipitates were weighed, purified according to the method of Berg, and Jaffe tests (11) performed.

Urorosein was determined qualitatively by two methods, the Ellinger-Dojmi or Beckh-Ellinger-Spies (B.E.S.) test (12) and the Nencki-Sieber (N.S.) test (13). Watson (14, 15) has shown that the color developing in the B.E.S. test is due to urorosein and not to porphyrins

as originally stated by Beckh, Ellinger, and Spies (12). The B.E.S. test was performed by adding two drops of two per cent sodium nitrite solution to ten ml. of urine and proceeding in a manner similar to that described by these authors. The N.S. test was carried out in the following manner. Ten ml. of urine were acidified with five ml. of twenty-five per cent hydrochloric acid. Two drops of two per cent sodium nitrite were added. The pigment was extracted with five ml. of iso amyl alcohol, and the mixture centrifuged.

Indican was determined by Obermayer's test. Equal parts of urine and Obermayer's reagent were mixed in a test tube, and the indican was then extracted with a small amount of chloroform.

RESULTS

"*Kynurenine*"—The urinary "kynurenine" excretion of a pyridoxine-deficient animal is indicated in Figure 1. It is clear that the "kynurenine" excretion varied with the tryptophane intake and that it diminished and finally disappeared on the addition of pyridoxine. It is of interest that at this time the animal was only mildly anemic, and it was true in general that the "kynurenine" excretion could not be correlated with other manifestations of pyridoxine deficiency such as the degree of anemia, the serum iron level or the severity or frequency of epileptiform convulsions.

Xanthurenic Acid—No positive tests for xanthurenic acid were obtained on the control urines (Table 1). All urine specimens from pyridoxine-deficient animals gave a positive test when the casein content of the diet was 26 per cent, as in the controls. The pigment first appeared between the eighteenth and the forty-sixth day of the deficiency. The average day of onset was the twenty-fifth. It was noted in traces at first, and gradually increased over a period of several weeks. Its appearance time could be correlated with the onset of anemia, but thereafter the amount present did not correspond with the degree of anemia, but rather with the dietary intake. If the dietary intake increased or if the ratio of casein in the diet was raised, the excretion of xanthurenic acid was increased. When zein, which in contrast to casein contains very little tryptophane, was substituted for the casein in the diet the pigment disappeared within two to three days. When tryptophane was added the pigment reappeared in two days. On the

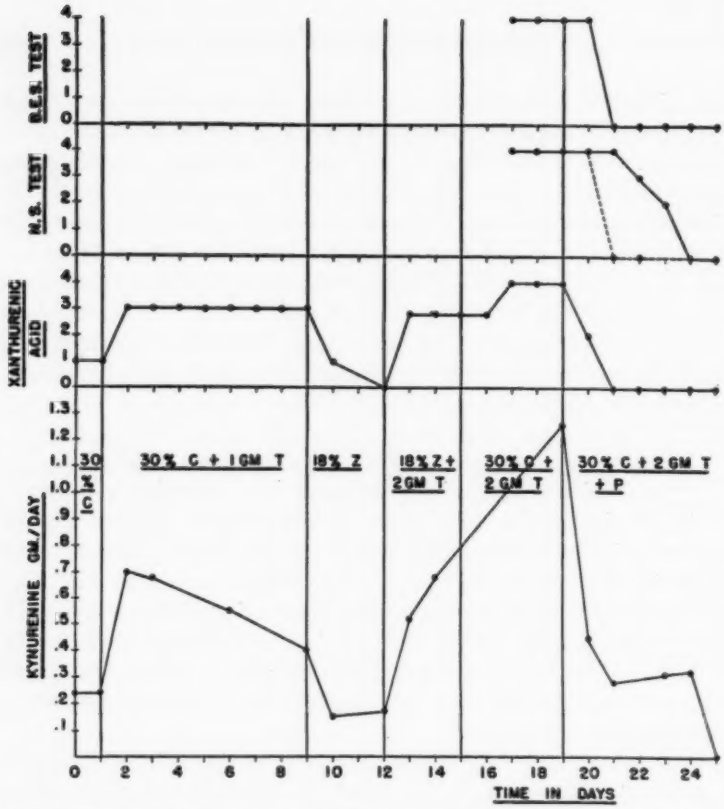

FIG. 1. EFFECT OF TRYPTOPHANE AND PYRIDOXINE ON THE EXCRETION OF FOUR TRYPTOPHANE DERIVATIVES IN THE URINE OF A PYRIDOXINE-DEFICIENT PIG

C. refers to casein, T to tryptophane, Z to zein and P to pyridoxine hydrochloride. Pyridoxine was given orally (1 mg. per kilo body weight daily) and intravenously (0.5 mg. per kilo daily).

The B.E.S. curve represents both the spontaneous reaction and the reaction with nitrite. The dotted line on the N.S. curve represents the spontaneous reaction, the solid line the reaction with added nitrite.

Note the increased excretion of "kynurenine" and of xanthurenic acid when tryptophane was added to a diet containing 30 per cent casein; the rapid decrease when zein, which is poor in tryptophane, was substituted for casein plus tryptophane; the increase in excretion when tryptophane was added and the still further increase when casein was substituted for zein. In the last period is shown the effect of pyridoxine in stopping the excretion not only of "kynurenine" and xanthurenic acid, but also of urorosein, as indicated by the B.E.S. and N.S. tests.

addition of pyridoxine to the diet the pigment disappeared entirely by the second day. These results are in harmony with those of Lepkovsky, Roboz, and Haagen-Smit (3) except that these authors found xanthurenic acid to disappear within six to twelve hours on the substitution of zein and gelatin in the diet or on the addition of pyridoxine. The conclusion is evident that xanthurenic acid excretion is dependent on tryptophane intake and is related to pyridoxine metabolism.

Kynurenic Acid—The excretion of kynurenic acid did not follow as clear a pattern as the excretion of xanthurenic acid. After two to four

TABLE 1

Relationship of Urinary Excretion of Xanthurenic Acid, Urorosein (N.S and B.E.S. tests) and Indican to Protein Content of Diet in Control and Pyridoxine-Deficient Pigs

	CONTROL ANIMALS	PYRIDOXINE-DEFICIENT ANIMALS		
	Dietary Casein, per cent			
	26	26	10	26 plus additional tryptophane
Number of Animals	9	8	4	4
Number of Specimens	33	24	8	17
Xanthurenic Acid, Average plus values	0	3	0	4
N.S. Test, Average plus values	1	3	1	4
B.E.S. Test, per cent positive	12	87	50	100
Indican, per cent positive	9	8	33	23

All animals were maintained on their respective diets for approximately six weeks before urine collections were begun. Twenty-four hour specimens were then taken at random intervals. When tryptophane was added to the diet it was in the form of *dl*-tryptophane (0.5 gm./pig/day).

months on the deficient diet, pyridoxine-deficient animals excreted an average of seven mg. of kynurenic acid per twenty-four hours in comparison with an average of three mg. in the urine of the control animals. Following the addition of tryptophane each day to the diet, the deficient animals excreted twenty-seven mg. and the control animals excreted thirty-one mg. The results were extremely variable and following purification of the Capaldi precipitate the yield was greatly reduced, and the melting points did not correspond with that of kynurenic acid. Jaffe tests on the purified product were either negative or only faintly positive. Our results suggest that the normal as

well as the pyridoxine-deficient pig excretes little or no kynurenic acid. It would seem that in the pyridoxine-deficient pig a fault in the metabolism of tryptophane is reflected by increased excretion of xanthurenic acid rather than kynurenic acid.

Urorosein—Upon acidifying the urine of deficient animals with sulfuric acid for the kynurenine determination it was observed that the color of the urine turned a deep rose red. A similar phenomenon was noted in 1882 by Nencki and Sieber (13) when they added strong mineral acid to the urine of a diabetic patient, and they named the new pigment urorosein. Later Herter (16) found that the chromogen of urorosein was indolacetic acid. This seemed interesting in view of the relationship between indole compounds and tryptophane, and led us to determine whether or not the red pigment which developed in pyridoxine-deficient urine is indolacetic acid.

Urines containing the red pigment were found to give positive N.S. and B.E.S. tests for urorosein. Furthermore, on the addition of dilute potassium hydroxide or ammonium hydroxide to the red amyl alcohol obtained from the N.S. test, the red color disappeared entirely and was quickly regenerated by shaking with a small quantity of twenty-five per cent hydrochloric acid. If the red amyl alcohol was mixed with several volumes of ether and shaken with a saturated aqueous solution of sodium acetate, the red color disappeared entirely. The same was true of the red acid layer obtained from the B.E.S. test. The red color was quickly regenerated by shaking with sufficient acid. The above properties correspond with those of urorosein and with those of pure indole-3-acetic acid obtained from Eastman Kodak Co.

In order to eliminate the possibility that the pigment was urobilin, urobilinogen, or bilirubin, the N.S. and B.E.S. tests were run on urine previously extracted with chloroform at pH 7, 4 and 3. It was found that the pigment was not removed by chloroform. Synthetic bilirubin failed to give positive N.S. and B.E.S. tests. Urobilinogen, urobilin, and bilirubin positive urines were obtained from humans and from dogs and these urines did not give positive B.E.S. or N.S. tests. Furthermore, quantitative urobilinogen and urobilin studies failed to show the presence of these pigments in many of the urorosein positive urines.

The possibility remained that the red pigment might be due to xanthurenic acid. Urine giving a strongly positive xanthurenic acid

test was acidified and the urorosein extracted as in the N.S. and B.E.S. tests. The extracted urine was then neutralized, ferric ammonium sulfate added and the green color of the xanthurenic salt was regenerated undiminished.

We have confirmed the observation of Layne and Watson (17) that crystalline kynurenic acid fails to exhibit a color in either the B.E.S. or N.S. test. Pure kynurenine failed to give positive tests.[3] Crystalline tryptophane also failed to give a positive B.E.S. or N.S. test. In order to eliminate the presence of porphyrins as an explanation for the color, the ether from a B.E.S. test was first extracted with five per cent hydrochloric acid and then with twenty-five per cent hydrochloric acid. Both solutions failed to exhibit the red fluorescence of porphyrins. This is in accord with the data of Watson (14, 15) that the red color of the B.E.S. test is not due to porphyrins, as was originally stated by Beckh, Ellinger and Spies (12).

It has been noted that synthetic indirubin colors the amyl alcohol red in the N.S. test (15). However, reduction to white indirubin by means of glucose and gentle heating in an alcoholic solution was not effected, indicating that the substance is not indirubin. Obermayer's test for indican was performed on the urines and found to be positive in only a small percentage. Most urines exhibiting urorosein reactions were found to be indican negative.

Absorption spectrum analyses[4] were made on the red amyl alcohol extract of the N.S. test and the red twenty-five per cent hydrochloric acid of the B.E.S. test. These curves were compared with those of normal pigs' urine giving negative N.S. and B.E.S. tests to which synthetic indole-3-acetic acid was added and which were then treated as for these tests. In acid solution the red pigment showed a well defined absorption band with a maximum at 520 mμ. Synthetic indole-3-acetic acid showed an exactly similar absorption under these conditions. The curves are shown in Figure 2. In amyl alcohol a strong band was exhibited with a maximum at 520 mμ. and a much weaker one with a maximum at 510 mμ. Again the analysis of indolacetic acid cor-

[3] We are indebted to Dr. C. P. Berg of the University of Iowa for kindly supplying us with crystalline kynurenic acid and pure l-kynurenine.

[4] We wish to express our appreciation to Dr. Buell of the Chemical Division for these determinations.

responded. The absorption bands obtained by us with urine and with pure indolacetic acid do not correspond with those obtained by Watson (15) (max. 554 mμ.) or those obtained by Meiklejohn and Kark (18) (max. 530 mμ.). The extent of oxidation of the substance, the presence of other substances and the method of spectral analysis could account for the disagreement. The important fact is that the substance ob-

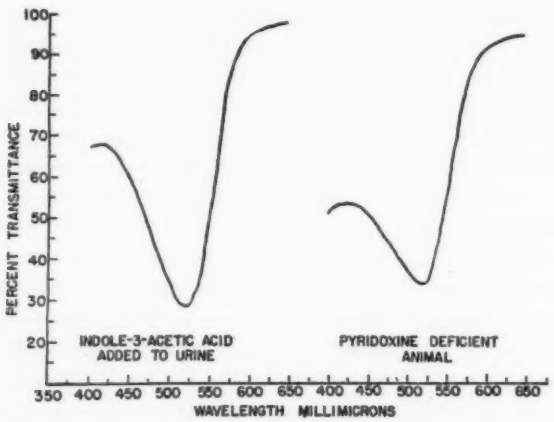

FIG. 2. ABSORPTION SPECTRA OF INDOLE-3-ACETIC ACID AND OF UROROSEIN

The curve on the left represents the spectral absorption of an acid solution of urine (B.E.S. test) to which indole-3-acetic acid was added. Prior to the addition of indole-3-acetic acid this urine gave a negative B.E.S. test and failed to exhibit a maximum at or near 520 mμ. The curve on the right represents the spectral absorption of an acid solution of urine (B.E.S. test) obtained from a pyridoxine-deficient animal giving a positive B.E.S. test. The determinations were made with a Coleman spectrophotometer.

tained from the urine of the deficient animals had a spectrum exactly similar to that of synthetic indolacetic acid under similar circumstances. Synthetic indole under similar conditions does not give a similar absorption spectrum.

From a consideration of the above data it was concluded that the red pigment developing in the urine of pyridoxine-deficient animals on the addition of strong mineral acid most probably was urorosein, the chromogen of which is either identical with or similar to indolacetic

acid. That the chromogen of the urorosein reaction obtained from the urine of pyridoxine-deficient pigs is indolacetic acid cannot be stated with certainty since it is known that the colors produced by glyoxylic acid with at least three different indole derivatives show apparently identical absorption maxima. Therefore, interference from other indole derivatives is possible, although it seems unlikely from the data presented.

Urorosein was determined by both N.S. and B.E.S. tests on a large number of urines from control and pyridoxine deficient animals. The results of qualitative tests are given in Table I. B.E.S. tests were not done quantitatively since the urine had been previously acidified and this appears to diminish the B.E.S. color. A small percentage of control urines exhibited faint and somewhat atypical reddish color in the amyl alcohol. This color was in all probability due to interfering substances rather than to urorosein. Positive B.E.S. tests in twelve per cent of the control animals might be explained on the basis that urinary urorosein comes not only from faulty tryptophane metabolism, but is also absorbed from the intestinal tract where it may be synthesized by bacteria. Other factors may also influence the excretion of urorosein.

By comparison with a standard indolacetic acid solution, urines of pyridoxine-deficient animals contain from 1 to 4 mg. per cent of indole pigment. This value is affected by the casein intake as can be seen in Table 1. If the casein in the diet is diminished the urorosein excretion correspondingly diminishes. Furthermore, if tryptophane is added the excretion is enhanced. To gain further evidence of this relationship a pyridoxine-deficient animal was given a diet consisting of fifty per cent casein. On the second day the N.S. and B.E.S. tests were three plus. The casein was then substituted by zein, a protein deficient in tryptophane, and by the third day of the zein diet the N.S. and B.E.S. tests had dropped to one plus. At this time 0.5 gm. of tryptophane was added to the zein diet daily. Four days later the B.E.S. test was three plus and the N.S. test was four plus. The effect of pyridoxine in causing the excretion of urorosein to cease in a deficient animal can be seen in Fig. 1. The N.S. and B.E.S. tests in this animal were done both with and without nitrite. When nitrite was not used, the reaction was termed spontaneous.

Toluene Soluble Pigments—Spies (29) and Watson (14) observed that the toluene layer of preserved urines from cases of pellagra turned pink or red on standing. Schwartz et al. (19) studied this toluene soluble pigment in detail and found by means of chromatographic analysis that it was composed of several pigments, two of which were similar to, but not identical with, synthetic indirubin. We found deep-red, orange, pink and yellow-red pigments in the toluene layer of preserved urines after they had stood for several months. This was observed in forty-one per cent of the pyridoxine-deficient urines and in fourteen per cent of the control urines. It was noted that the same urines had been previously strongly acidified. Absorption spectra on the deep-red toluene showed a maximum at 520 mμ. with a much weaker band at 510 mμ. This was identical with the spectrum of urorosein. The orange, yellow-red and pink pigments were not studied.

DISCUSSION

The data presented suggest that the urinary excretion of "kynurenine," xanthurenic acid and urorosein is related to the amount of tryptophane in the diet, and that in pyridoxine-deficiency the excretion of these substances is increased. In the pyridoxine-deficient animals it is not likely that the urorosein arose through putrefaction of tryptophane by the intestinal bacteria since the incidence of positive tests in these animals was much greater than in the control animals and there was no evidence of increased intestinal stasis or putrifaction in the deficient group. Also against this possibility is the fact that indicanuria was not increased in the pyridoxine-deficient animals as one would expect if the urorosein in the urine came from increased intestinal putrefaction. Furthermore, when pyridoxine was given either orally or intravenously the urorosein disappeared from the urine before there was any improvement in the general condition of the animals. It cannot be concluded that the urorosein originated from the *d* fraction of the *dl*-tryptophane for when the animals received a diet containing 26 per cent casein and no added tryptophane, 87 per cent of the urines were urorosein positive. That urorosein is derived from the natural isomer is shown by the fact that when the dietary casein was decreased to 10 per cent the incidence of positive tests diminished and finally when zein was substituted for the casein the urines failed to give urorosein reactions.

The precise relation between tryptophane and pyridoxine metabolism is obscure. It has been shown by others that kynurenine (20), kynurenic acid (21) and xanthurenic acid (22) are excretory products of tryptophane metabolism and are devoid of growth promoting properties when added to a diet deficient in tryptophane. It would seem then that in pyridoxine-deficiency tryptophane is not being metabolized in the usual manner, and one might expect the pyridoxine-deficient animal to be tryptophane deficient as well. This gives rise to the question whether the anemia of pyridoxine deficiency may be due to a lack of utilization of tryptophane, since a deficiency of tryptophane alone is known to produce anemia. Details pertaining to this question are being published separately.

It is of interest that there is a marked species variation in the excretion of the tryptophane metabolites. The rat and the rabbit are known to produce xanthurenic acid, kynurenic acid and kynurenine (1). Dogs excrete large quantities of kynurenic acid and kynurenine but little xanthurenic acid (23, 1). We have presented evidence that the pig forms "kynurenine" and xanthurenic acid but excretes little or no kynurenic acid. It has been demonstrated that man excretes kynurenine (9) but not xanthurenic acid (24). There is disagreement as to whether kynurenic acid is excreted by man (25, 26).

Urorosein has been shown to be present in human urine in a variety of conditions. Nencki and Sieber (13) noted positive tests in ten per cent of all pathological urines. Vilter, Vilter and Spies (27) noted the B.E.S. test to be positive in one of two cases of severe diabetic acidosis and reported that this test became negative following treatment of the acidosis. Watson and Layne (28) reported the test positive following the addition of nitrite in seven of eight cases of diabetic acidosis. Meiklejohn and Kark (18) and Watson and Layne (28) reported its presence in urine obtained from patients with pellagra. The latter authors were unable to correlate its presence or absence with the degree of nicotinic acid deficiency or with the administration of this vitamin. They found, furthermore, that the spontaneous reaction was consistently negative in urine specimens from dogs having experimental blacktongue. It seems possible, therefore, that the presence of urorosein in the urines of pellagrins is due to pyridoxine deficiency. It would not be expected that uroroseinuria, in conditions other than nutritional deficiency, is due to altered pyridoxine metabolism and this

is in keeping with our recent studies on humans.[5] We have frequently found urorosein in the urine in a variety of pathological states and in these we have been unable to affect its excretion by administering pyridoxine either orally or intravenously.

It may be stressed that the evidence presented in this paper is limited and is largely presumptive. However, it seems evident from the data already obtained that altered tryptophane metabolism is a manifestation of pyridoxine deficiency. Studies on the quantitative relationships of the various tryptophane derivatives using *l*-tryptophane, the natural isomer, are now indicated.

SUMMARY

The occurrence of three excretory products of tryptophane metabolism, "kynurenine," kynurenic acid and xanthurenic acid in the urine of pyridoxine-deficient swine has been studied. Evidence is presented which indicates that the excretion of "kynurenine" and xanthurenic acid is increased in such animals and is related to the amount of tryptophane in the diet. Excretion of these substances ceases on administration of adequate amounts of pyridoxine.

The results of measurements of kynurenic acid in the urine of normal as well as pyridoxine-deficient animals gave equivocal results. The data obtained suggest that in the pyridoxine-deficient pig a fault in the metabolism of tryptophane is reflected by increased excretion of xanthurenic acid rather than kynurenic acid.

In the urine of pyridoxine-deficient animals a red pigment was observed to develop on the addition of strong mineral acid. Data are presented which suggest that this is urorosein. The excretion of this pigment, like that of "kynurenine" and of xanthurenic acid appeared to be related to the intake of tryptophane and disappeared on the administration of pyridoxine.

These observations suggest that in pyridoxine deficiency tryptophane is not metabolized in the usual manner.

It is suggested that the presence of urorosein in the urine of pellagrins may be due to pyridoxine deficiency but it is noted that this pigment is found in human urine under circumstances which appear to be unrelated to pyridoxine deficiency.

[5] Jones, P. J., and Cartwright, G. E., unpublished data.

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THE DIAGNOSIS OF INFLUENZA VIRUS INFECTIONS BY THE AGGLUTINATION-INHIBITION TEST [1]

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The purpose of this survey is the presentation of serological evidence for the diagnosis of influenza A virus infections during an epidemic of respiratory diseases in Baltimore, Maryland, during December, 1943. The agglutination-inhibition reaction used in this investigation was first described by Hirst in 1941 (1), and it has greatly simplified the technique of influenza virus studies. An evaluation of the reliability of this test is presented. Although the clinical application of the serum-inhibition reaction has not been sufficiently extensive to permit its final appraisal, this test is likely to become a serological method of great diagnostic value in the hospital laboratory.

CLINICAL PICTURE

During December, 1943, an epidemic of respiratory infections occurred in Baltimore, Maryland. This increased prevalence of clinically diagnosed cases of "grippe," "flu," and "colds" was part of a nationwide epidemic of mild influenza. Reports of an increased incidence of influenza came first from Michigan, Ohio, and Wisconsin during the week ending November 13, 1943 (2, 3). Rapidly infection spread to the East and to the West. By the middle of December the epidemic wave reached the Pacific. After the first week in January there was a marked decrease in the number of reported cases. During December, 1943, and January, 1944, 578,000 cases of clinical influenza were reported in the United States. This epidemic arose, spread throughout the country, and subsided during a period of seven weeks.

The infections were mild with few complications or hospital admissions. The clinical picture of a typical illness follows:

[1] This study was aided by a grant from the Rockefeller Foundation Fluid Research Fund.

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A 26-year old medical student was admitted to The Johns Hopkins Hospital on December 17, 1943, with the complaints of headache and muscle pains of 12 hours' duration. Suddenly, on the morning of admission, he developed chilliness, fatigue, and mild headache. Lumbar backache and pains in the leg muscles soon followed. He developed a non-productive cough.

His temperature was 102°F. He was acutely ill with a flushed face and moderate photophobia. There were no positive physical findings in the chest or abdomen. On the following day his temperature rose to 103°F., then returned to normal on the third day. He felt tired, but otherwise well. On the fifth day after the onset of his illness he returned to his duties.

During this epidemic period serological studies were performed on a small group of nurses and medical students with influenza. An attempt was made to establish the etiology of these infections by the agglutination-inhibition test.

Several epidemics of clinical influenza have been studied since the viruses of influenza A and B were first isolated. The following observations have been established: An epidemic may be due to influenza A virus, influenza B virus, both A and B viruses, or to a completely unknown etiological agent or agents (4, 5). The clinical pictures produced by the known and unknown infections are not specific, and they do not permit one to make a definite diagnosis (5, 6). Thus, the etiological diagnosis of influenza A or B virus infection depends upon either the isolation of the virus from the patient's throat washings or the detection of a rise in specific antibodies in the patient's blood during convalescence.

Influenza virus can be isolated from throat washings of patients by the amniotic inoculation of chick embryos and by the intranasal inoculation of ferrets. The standard procedure for the detection of a rise in neutralizing antibodies is the mouse protection test. In nearly every case in which influenza virus has been isolated from a patient's throat washings, the convalescent serum has shown a rise in neutralizing titer. Antibodies to influenza A and B virus develop in 5 to 14 days after the onset of illness. These methods of diagnosis are quite reliable, but they cannot be easily adapted to hospital use.

A great simplification in method for the study of influenza virus infections has been made by Hirst (7). He has devised a reliable method for the measurement of agglutination-inhibiting antibodies. This test will probably enable one to detect influenza A or B infections by a comparatively simple technique.

Influenza virus agglutinates mammalian and fowl red blood cells *in vitro* and produces a visible reaction which can easily be measured. This is called the hemagglutination titration, or the chick red cell agglutination test. Immune sera inhibit this reaction, and the degree of this inhibition can also be ascertained. The titration of sera by this method has been termed the agglutination-inhibition test or the serum-inhibition test. This measurement of inhibiting substances in serum represents a determination of the antibody titer against influenza A or B virus.

HEMAGGLUTINATION REACTION

The *method* used by the authors in performing the agglutination titration is essentially the same as that described by Hirst (7, 8). This will be summarized together with the various modifications of the test made by others. This reaction involves two reagents (red cells and virus suspensions) with one variable (serial virus dilutions).

Virus suspensions of pooled allantoic fluid from infected chick embryos were used. The PR8 strain of influenza A and the Lee strain of influenza B were employed.[3] These were stored at 4°C. Chicken red blood cells were prepared in 1.5 per cent saline suspensions.

Serial two-fold dilutions of virus in saline were made in smallbore serological tubes (0.9 cm. in diameter). To 1 cc. of each dilution was added 1 cc. of the 1.5 per cent red cell suspension. The tubes were then shaken. After standing 75 minutes at room temperature the titration was read. The degree of red cell agglutination was measured by comparing the densities of the supernatant fluids in the various dilutions with the densities of standard red cell suspensions. The dilution of virus which produced a 50 per cent diminution in the number of red cells in suspension was chosen as the end-point. This was considered as one agglutinating unit of virus suspension.

A number of *variations in method* have been described. The test has been done with human red cells, group O, guinea pig cells, and others (9, 10). Variable concentrations of cells (2, 1.5, 0.25, and 0.07 per cent) have been employed (7, 11). Hirst and Pickels (12) have devised a densitometer to increase the accuracy of reading the end-point. However, this is not required in serological studies. The

[3] The virus strains used in these tests were kindly supplied by Dr. W. M. Stanley, Rockefeller Institute for Medical Research, Princeton, N. J.

end-point of the titration may also be read by using the agglutination in the bottom of the tube as an indicator (13) instead of the density of the supernatant suspension. The authors have found that most of these variations in method are equally satisfactory.

The available evidence suggests that this agglutination reaction measures the titer of active or inactivated influenza virus. This is based on the following observations:

1) The rises in titer of the virus and of the agglutinating substances in the allantoic fluid of the chick embryo after inoculation are parallel phenomena (14). As a result of this fact, the infectivity titer of fresh allantoic fluid parallels the agglutination titer (7).

2) When the agglutinating agent is adsorbed to red cells, the virus is also adsorbed. Similarly, the infective particles and the agglutinating substances are eluted simultaneously (15).

3) In the ultracentrifuge, the curves of sedimentation of virus and agglutinating titers are similar (16).

4) The agglutinating activity and the infectivity of a virus suspension are both specifically neutralized by immune sera.

The only significant difference between the agglutinating and infective particles is their varying susceptibility to physical and chemical agents. The virus may be completely inactivated by formalin, heat, storage, or ultraviolet light, and yet the agglutinating titer may be maintained. Thus, although these two agents are not identities, the infective activity and the agglutinating activity in a virus suspension are very closely associated and may be fractions of the same particle. This close correlation of the hemagglutinating and infective titers suggested to Hirst the use of the inhibition reaction as a method of measuring the antibody titer of serum.

AGGLUTINATION-INHIBITION TEST

The *method* used by the authors in performing this reaction is that of Hirst (7). In this test there are three reagents (red cells, virus, and serum) with one variable (serial dilutions of serum).

In a preliminary agglutination titration the dilution of virus containing one agglutinating unit was determined. Four units were used in each tube in the inhibition test.

Sterile blood sera from patients were collected, inactivated at 56°C., and stored

at −10° C. Since only a significant increase in antibody titer is of diagnostic value, at least two blood specimens from each patient were required. The first serum was drawn during the first week of illness, and a second sample was taken from two to six weeks after onset. All blood specimens collected from one patient were tested at the same time with the same virus suspension and red cells. This was essential in order to obtain repeatable results. Serum dilutions were expressed in terms of the final concentration.

The sera were diluted in two-fold steps in saline. To 0.5 cc. of each dilution was added 0.5 cc. of virus suspension containing four agglutinating units. To this mixture was added 1 cc. of a 1.5 per cent red cell suspension, and the tubes were shaken. The end-point of the inhibiting power of the serum was read after 75 minutes at room temperature. This was determined with standard red cell suspensions. As a further check the end-point of agglutination in the bottoms of the tubes, after all the cells had sedimented, was also read. The titration of an immune rabbit serum was used as a control. In addition, the agglutination reaction was repeated.

This procedure can be readily adapted to use in the serology laboratory of a hospital. The virus suspensions and immune sera are quite stable at ice-box temperature, and they could be supplied by a biological firm. Human, group O, red blood cells can be employed to avoid the need of obtaining chicken cells.

EVALUATION OF THE SERUM-INHIBITION REACTION

The value of the inhibition test in the study of influenza virus infections depends upon what the titration actually measures and the accuracy of this measurement. Several investigators have studied sera from influenza A epidemics by both agglutination-inhibition and by mouse protection tests. McClelland and Hare (17) reported a group of eleven cases with significant rises in titer by both methods of testing. Hirst (7) showed a close quantitative correlation between these two methods in a study of 16 acute and 21 convalescent influenza sera. Others have confirmed this observation, and their results are summarized in Table 1. Of 27 sera with a significant rise in neutralizing antibodies, 26 showed a corresponding rise by the *in vitro* test. This evidence shows that the inhibition test with influenza A virus measures either neutralizing antibody or something which fairly consistently parallels neutralizing antibody.

In regard to influenza B virus, similar studies have not been done. No attempt has been made to correlate the rise of serum-inhibition

titer against influenza B with the rise of mouse-protective antibody titer in clinical cases.

An important negative finding is the establishment of the fact that serum-inhibition titers against influenza A and B remain relatively unchanged during the course of a variety of other infectious diseases. Any rise in titer which might occur in response to other infections would decrease the specificity of this diagnostic method. Stuart-Harris (20) tested 52 pairs of sera from sporadic respiratory infections

TABLE 1

Influenza A infections—Correlation of serum-inhibition and mouse protection tests

AUTHOR	MOUSE-PROTECTION: NO. OF CASES WITH SIGNIFICANT RISE	SERUM-INHIBITION: NO. OF CASES WITH FOUR-FOLD RISE
McClelland, Hare, et al. (17, 18)	18	17
Bodily and Eaton (19)*	8	8
Stuart-Harris (20)†	1	1
Total	27	26

* Tests run with PR8 strain.

† Tests run with pooled sera.

TABLE 2

Control sera studied by agglutination-inhibition reaction

AUTHOR	DIAGNOSIS	NO. OF PAIRS OF SERA	FOUR-FOLD RISE IN TITER	
			Influenza A	Influenza B
Stuart-Harris (20)	Respiratory infections	52	0	0
Hare, et al. (21)	Respiratory infections	55	0	0
Authors	Miscellaneous infections	76	0	0

by the *in vitro* test. In none of these cases was there a four-fold rise in titer to influenza A or B (Table 2). Six pairs of these sera were tested by mouse protection tests with negative results. Five throat washings from the same group of cases were tested for influenza virus with no positive findings. Fifty-five pairs of sera from two epidemics of respiratory infection in October, 1941, and October, 1942, were studied by Hare, Hamilton, and Feasby (21). None of these sera showed a four-fold rise in titer to influenza A or B.

From January to April, 1944, the authors studied 76 pairs of sera from patients with a variety of infectious diseases. Cases of sporadic respiratory infections, pneumonia, measles, mumps, chicken pox, scarlet fever, and meningitis were studied. This group represents a control group for the study of the usual variation in the inhibiting titer of an individual's serum against influenza virus. The acute phase blood specimens were all drawn during the first week of illness, and the convalescent ones were collected from two to six weeks after onset of the disease. The results are summarized in Table 3. In none of these pairs of sera was a four-fold or greater rise in titer demonstrated

TABLE 3

Variation of inhibition titers of control sera

DIAGNOSIS	NO. OF PAIRS OF SERA	INFLUENZA A		INFLUENZA B	
		No. with two-fold rise	No. with four-fold rise	No. with two-fold rise	No. with four-fold rise
Upper respiratory infections	22	2	0	0	0
Pneumonia					
Pneumococcal	11	0	0	2	0
Other	12	0	0	0	0
Measles	9	0	0	0	0
Mumps	10	2	0	1	0
Chicken Pox	3	0	0	0	0
Scarlet Fever	7	1	0	1	0
Meningitis meningococcic	2	0	0	1	0
Total	76	5	0	5	0

against either influenza A or B. A two-fold increase in titer occurred in 5 sera (6.5 per cent) tested against influenza A and also in 5 sera tested against influenza B. Twenty-two patients with sporadic respiratory infections occurring after the influenza epidemic period were included in this group. In none of these cases was there a sigficant rise in influenza A or B titer.

The majority of convalescent human sera, which show a significant rise in the inhibition test to one influenza A strain, also show an elevation to a variety of A strains. However, this is not true in every case. Bodily and Eaton (19) tested forty pairs of sera with four different influenza A strains and found that the number of four-fold rises varied

from 87 per cent with the PR8 strain to 60 per cent with the swine virus. No reports of variation in antibody titer with different strains of influenza B have appeared.

With repeated titrations of a blood sample a technical error of one-half a tube may occur. In addition, two sera collected at a two-to-four week interval from a patient with an entirely different infection may have a repeatable increase of one tube (two-fold) in titer. Therefore, a four-fold or greater rise in influenza antibody is required before the test is of any diagnostic significance.

Thus, the serum-inhibition test has proved so far to be an accurate measure of the neutralizing antibody titer for influenza A and probably for influenza B infections. Certain limitations are to be recognized. Occasionally, sera do not show parallel rises in titer to a variety of influenza A strains. In a few cases of influenza infection the antibody response is not of sufficiently large dimensions to produce a four-fold rise. These factors occasionally produce false-negative results. However, the test is of definite diagnostic value in the identification of the virus etiology of an epidemic outbreak.

APPLICATIONS OF THE INHIBITION TEST

The application of this test to the diagnosis of *epidemic influenza* was first shown by Hirst (8). He reported studies on 232 pairs of influenza A sera. The average titer of the acute sera was 104, and of the convalescent sera, 865. This represents an average rise of eight-fold. Other workers have described similar increases in titer to influenza A virus with an average rise of eight to ten-fold. Their results are summarized in Table 4. The inhibition test has also been used as a diagnostic aid in influenza B infections by Hare and his associates (21, 22). In a serological study of 163 patients in Canada during an epidemic of respiratory infections, 48 pairs of sera showed a four-fold or greater rise in titer to influenza B. The average increase in titer was ten-fold.

The serological response to *experimental infection*, produced by the intranasal inoculation of recently isolated influenza A virus, has been measured by the inhibition reaction. In one investigation (23) 11 persons contracted influenza among an inoculated group of 72 individuals. These 11 cases showed, by the *in vitro* test, a significant anti-

body response to infection. In a second study (24) seventeen individuals were inoculated. Four developed clinical influenza, and thirteen showed an antibody response in their sera. Francis and his associates (11) sprayed a virulent suspension of the Lee strain of influenza B virus into the throats of 30 subjects. Of this group, 27 developed fever above 100°F. (R), and 26 of the 30 individuals showed a four-fold or greater elevation in antibody titer by the agglutination-inhibition reaction. Twenty-four of these same patients were reinoculated intranasally four months later with influenza B virus. On this occasion 21 of 24 showed a temperature above 100°F. (R), and eight showed a further four-fold rise in antibody titer.

TABLE 4

Serological diagnosis of influenza A infections by inhibition test

AUTHOR	NO. OF PAIRS OF SERA	PER CENT OF CASES WITH SIGNIFICANT RISE TO A	AVERAGE NO. FOLD RISE
Hirst (8)	232	—	8
Bodily and Eaton (19)	40	87	10
Burnet (13)	30	97	—
Stuart-Harris (20)	1	—	24
Hare, et al. (18)	27	—	12
Authors	14	71	6

The serum-inhibition test has been widely used to study the antibody response after *vaccination* with influenza A and B. After subcutaneous inoculation with concentrated virus preparations a four to ten-fold rise in the average antibody titer occurs (8, 19). The antibody response reaches a peak in two weeks and subsides over a period of months. After intranasal inoculations of living, attenuated virus A and B, 10 to 30 per cent (25, 26) show a significant increase in the *in vitro* titer. Thus, the inhibition test is a very useful method in the evaluation of the protection against influenza afforded by vaccines.

RESULTS OF PRESENT INFLUENZA STUDY

The authors studied a group of fourteen nurses and medical students hospitalized during the epidemic period of December, 1943. These patients presented similar clinical pictures. The onset of illness was

sudden with fever, chilliness, and lethargy. Coryza and sore throat were present in some of these cases. Pain in the leg muscles, backache, and pain behind the eyes were common symptoms. The maximum temperatures in these patients varied from 101 to 105°F. orally, and the febrile period ranged from two to four days. No complications developed in any of this group.

In 10 of the 14 pairs of sera tested there was a four-fold or greater increase in the *in vitro* level (Table 4). The average rise in agglutination-inhibition titer was six-fold. The influenza A titers of the acute and convalescent sera in the ten cases with significant elevations are recorded in Figure 1. None of these fourteen pairs of sera showed a significant rise to influenza B virus.

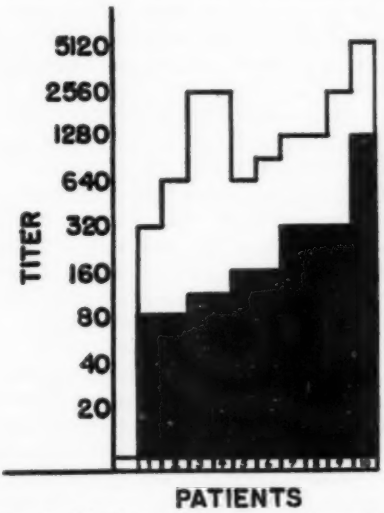

FIG. 1. Influenza A infections. Titers of acute and convalescent sera. Solid columns indicate titers of acute sera, and open columns those of convalescent sera.

SUMMARY

1) During December, 1943, a widespread epidemic of mild clinical influenza occurred in Maryland and throughout the United States.

At this time, a serological diagnosis of influenza A infection was made on a group of representative patients by the agglutination-inhibition reaction.

2) Between January and April, 1944, 76 pairs of control sera from a variety of infectious diseases were studied. Included in this group of cases were 22 patients with sporadic respiratory infections occurring during the four months after the epidemic period. In none of these cases was there a significant rise in the inhibition titer to influenza A or B.

3) A tentative evaluation of the serum-inhibition test and its applications to the study of epidemic influenza are presented.

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COLD HEMAGGLUTINATION IN PRIMARY ATYPICAL PNEUMONIA[1]

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The purpose of this survery is to report the results of cold agglutination studies with a group of sera taken from patients with primary atypical pneumonia and other illnesses during the winters of 1942–43 and 1943–44. The results of similar studies by other investigators are summarized, and a tentative evaluation of cold agglutinin determinations in the diagnosis of primary atypical pneumonia is presented.

PREVIOUS INVESTIGATIONS

A complete review of the subject of cold hemagglutination has recently been presented by Stats and Wasserman (1). Cold agglutinins in low dilutions (1:1 to 1:5) are present in the sera of most normal persons. High titers occur in some cases of paroxysmal cold hemoglobinuria, Raynaud's syndrome and hemolytic anemia. In these cases they are probably of pathogenetic importance. Trypanosomiasis is the only infectious disease (with the exception of atypical pneumonia) in which cold agglutinins are commonly present. In addition, high titers occasionally appear in sera of patients with leukemia, cirrhosis of the liver, multiple myeloma, and other diseases.

The frequent occurrence of cold agglutinins in primary atypical pneumonia of unknown etiology was first described in 1943 by Peterson, Ham, and Finland (2). They observed that "the great majority" of cases had titers of 1:10 to 1:10,000. It was suggested that this reaction might offer a criterion for the differentiation of primary atypical pneumonias until definite etiological agents had been established. Four other investigators (3, 4, 5, 6) have studied cold agglutinins in the sera of a total of 293 patients with atypical pneumonia in order to establish the diagnostic significance of this method. The results of these authors are summarized in Table 1. Each investigator selected

[1] Aided by a grant from the Rockefeller Foundation Fluid Research Fund.

[2] John D. Archbold Fellows in Medicine.

a minimum dilution which represented arbitrarily the borderline between a normal and an abnormal titer. Two authors selected a dilution of 1:32, one investigator chose a titer of 1:20, and the fourth author considered a dilution of 1:8, as the minimum significant titer. On the basis of these criteria 33 to 81 per cent (an average of 55 per cent) of the cases of atypical pneumonia had significant cold agglutinins. Nearly half of the total number of patients showed no rise in titer during their illnesses. When cold agglutinins did develop, they usually appeared about one week after the onset of illness. They remained elevated for two to three weeks and subsided in four to six weeks after the onset. The maximum titers usually varied from 1:40 to 1:320. Rarely, agglutinins in a dilution of 1:10,000 or higher were observed. Over 100 control sera from patients with other ill-

TABLE 1

Primary Atypical Pneumonia Occurrence of Cold Hemagglutinins in Reported Cases

AUTHORS	NO. OF CASES	PERCENTAGE WITH POSITIVE COLD AGGLUTININS
Horstmann and Tatlock (3)	43	63
Turner et al. (4)	83	53
Meiklejohn (5)	74	81
Commission, Fort Bragg (6)	93	33
Total	293 Cases	Average: 55 per cent

nesses were also studied by each of these four investigators. Significant titers occurred in 0 to 4 per cent of these sera.

PRESENT INVESTIGATION

During the winters of 1942–43 and 1943–44, 429 sera were collected from 240 patients admitted to the Johns Hopkins Hospital with various infectious diseases. Included in this group were 50 patients on whom discharge diagnoses of primary atypical pneumonia of unknown etiology were made. These diagnoses were based on clinical picture, x-ray findings, blood and sputum examinations. Cold agglutination reactions were performed on all these sera in order to determine the incidence of high agglutinin titers in primary atypical pneumonia and in other infectious diseases.

MATERIALS AND METHODS

Sera. Sterile blood specimens were collected from patients and allowed to clot at room temperature. After centrifugation the sera were separated and stored at −10°C. Whenever it was possible, samples were tested on the day of collection.

Red Blood Cells. Human, group O, red cells, obtained from one of three different individuals, were washed in saline and prepared in a 2 per cent suspension. Only cells less than one day old were used in the test.

Cold Agglutination Titration. Serial two-fold dilutions of serum were made. To 0.5 cc. of each dilution was added 0.5 cc. of the red cell suspension. Control tubes containing 0.5 cc. of saline and 0.5 cc. of cell suspension were also prepared. The tubes were shaken, placed in an ice bath at 0° to 5°C. overnight, and read on the following morning. The degree of agglutination in the bottoms of the tubes was graded from 4+ to 0. The highest dilution of serum, which produced definite, macroscopic agglutination, was chosen as the end-point. Titers were expressed in terms of the serum dilution before the addition of red blood cells. The sera were then left at room temperature for an hour to demonstrate that the cold agglutinins were reversible.

TABLE 2

Incidence of Significant Cold Agglutinin Titers in Atypical Pneumonia and in Controls

CASES	NO. OF CASES	NO. OF SERA	PERCENTAGE OF CASES WITH AGGLUTININ TITERS OF 1:40 OR HIGHER
Atypical pneumonia	50	122	40.0
Controls	190	307	3.7

RESULTS

Cold agglutinin determinations were performed on 122 sera from 50 patients with atypical pneumonia (Table 2). In order to evaluate the results of these studies, a positive cold agglutinin titer of 1:40 was arbitrarily chosen as the minimum significant dilution. On the basis of this criterion, abnormally high titers occurred in 40 per cent of the patients with atypical pneumonia, and in 3.7 per cent of the control cases.[3] The maximum titers obtained in the 50 cases of atypical pneumonia are presented in Table 3. In 20 patients with positive cold

[3] If a dilution of 1:20 were chosen as the minimum significant level instead of 1:40, then the incidence of cold agglutinins would be 46 per cent in the atypical pneumonia cases and 11 per cent in the controls. Fourteen of the control cases and three of the atypical pneumonia group had maximum titers of 1:20 (Table 3).

agglutinins, the levels varied from 1:40 to 1:640. In several cases the rise and fall in agglutinins during the course of the infections were followed. It was observed that the maximum levels were reached in one to three weeks after the onset of illness. During the subsequent two to four weeks the serum titers usually fell to normal. This rise and fall in titer in atypical pneumonia is illustrated in Figure 1 by the sera of the patient, S. P. However, the time at which agglutinins

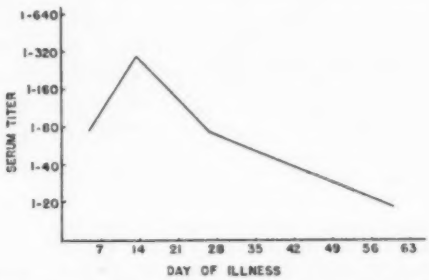

FIG. 1. COLD AGGLUTININ IN PATIENT S. P., ATYPICAL PNEUMONIA

TABLE 3

Maximum Cold Agglutinin Titers in Atypical Pneumonias and in Controls

CASES	NO. OF CASES	NO. OF CASES WITH MAXIMUM TITERS OF						
		<1:20	1:20	1:40	1:80	1:160	1:320	1:640
Atypical pneumonia.....	50	27	3	6	6	6	1	1
Controls.....	190	169	14	4	2	0	0	1

develop and disappear is variable. In one patient a titer of 1:40 was still present 81 days after the onset of her illness.

During the winter of 1943-44, 307 control sera from 190 patients with a wide variety of illnesses were tested for cold agglutinins. Titers of 1:40 or higher occurred in 3.7 per cent of these cases (Table 2). The maximum titers are presented in Table 3. Four patients developed agglutinins in 1:40 dilutions; and two, in 1:80 dilutions. One patient showed a rise in titer to 1:640 with subsequent fall during the course of pneumococcal pneumonia, type 12. The various diseases included in the control group were divided arbitrarily into two parts: (a) re-

spiratory illnesses, which might possibly be considered in the differential diagnosis of atypical pneumonia, and (b) other illnesses clinically unrelated to atypical pneumonia. The number of cases of each disease studied is listed in Table 4. Among 104 various respiratory illnesses two patients (1.9 per cent)developed significant cold agglutinins. One of these patients had an upper respiratory infection with beta-hemolytic streptococci in her throat, and the other patient had pneumococcal pneumonia. Among a group of 86 patients with measles,

TABLE 4

Incidence of Cold Agglutination in Control Cases

DIAGNOSIS	NO. OF CASES	PERCENTAGE OF CASES WITH TITERS OF 1:40 OR HIGHER
A. Respiratory illnesses	104	1.9
Pneumonia	—	—
Pneumococcal	33	3
Other	17	0
Upper respiratory infections	33	3
Influenza A*	10	0
Asthma	8	0
Tuberculosis	3	0
B. Others	86	5.8
	—	—
Measles	11	9
Mumps	14	0
Scarlet fever	8	0
Syphilis	33	12
Miscellaneous	20	0

* Diagnosis based on hemagglutination-inhibition reaction (Hirst).

mumps, scarlet fever, syphilis, and other infections, 5 cases (5.8 per cent) had high cold agglutinin titers. Four of these patients had syphilis.

DISCUSSION

Since atypical pneumonia is a disease of unknown etiology or etiologies, it is obviously impossible to ascertain whether or not cold agglutinins are specific antibodies. However, the fact that cold agglutinins in high titer occur in a variety of other diseases makes this hy-

pothesis unlikely. In addition, the inconstant occurrence of cold agglutinins in atypical pneumonia cases also makes this conception less tenable.

The value of this reaction as a diagnostic criterion depends upon upon the incidence with which it occurs in atypical pneumonia. Since significant titers of cold agglutinins develop in only half of the cases with this clinical picture, this test is not of diagnostic value in the individual case. However, cold agglutinins are of some confirmatory value in the diagnosis of atypical pneumonia. A high titer of cold agglutinins in the serum of a patient with the clinical picture of atypical pneumonia does confirm the diagnostic impression. In this respect the test is of definite significance. The final evaluation of this reaction must await the elucidation of the etiology or etiologies of this infection.

SUMMARY

1. In a group of 50 patients with primary atypical pneumonia cold agglutination titers in 1:40 or higher dilutions occurred in 20 cases (40 per cent). In a control group of 190 patients with a variety of infectious diseases 7 cases (3.7 per cent) had significant agglutinin titers in their sera.

2. The cold agglutination reaction is a simple laboratory procedure of confirmatory value in the diagnosis of some cases of primary atypical pneumonia of unknown etiology. It is not of definite diagnostic value in the individual patient.

The authors wish to express their appreciation to Miss Helen Deitz, Mr. James Johnson, and Miss Patricia Lamdin for their assistance in this study.

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BOOK REVIEWS

(These reviews represent the individual opinions of the reviewers and not necessarily those of the members of the Editorial Board of this Journal.)

Oral Pathology. A Histological, Roentgenological, and Clinical Study of the Diseases of the Teeth, Jaws, and Mouth. By KURT H. THOMA. 2nd ed. 1295 pp. Illus. $15.00. The C. V. Mosby Company, St. Louis, 1944.

This book, written primarily for the dental profession, should be as well a standard item in the consulting library of physicians, surgeons, pediatricians, and pathologists. It contains a wealth of information, logically presented, not only about primary disorders of the teeth and surrounding tissues, but also depicts in detail the effects of systemic diseases on these various structures.

There are two major points of criticism. The first, and less important, of these is that this second edition has scarcely been revised at all. In the three years that have elapsed since the first publication considerable advances have been made in many of the fields discussed, yet with three or four exceptions the texts of the two editions are identical. This is the more to be regretted since the work is, for the most part, an excellent and valuable one.

The second major defect in this work is that the selection of material from the literature and its discussion is often quite uncritical. This fault is most clearly apparent in the chapters and sections dealing with the effects of vitamin deficiencies and endocrine disorders. For example: two pages are devoted to a factual report of the work of Rowntree and his associates on the growth-promoting activity of thymus extracts. No critical comments are made and nothing is said of the fact that numerous other investigators have failed to confirm these results. Where dealing with subjects of which the author obviously has more personal knowledge, however, the text is authoritative.

The work is carefully and beautifully illustrated. The color plates are excellent.

J. E. G.

Female Endocrinology. By JACOB HOFFMAN. 788 pp. Illus. $10.00. W. B. Saunders Company, Philadelphia and London, 1944.

This volume is primarily concerned with the clinical aspects of endocrinology with special reference to the reproductive system. The book is divided into three parts: Physiology (290 pages), Clinic (357 pages) and Laboratory (84 pages). The endocrine disturbances most often encountered in gynecological practice are excellently presented. The author maintains a conservative and critical outlook in the management of endocrine disorders. The clinical literature is reviewed critically and exhaustively. Laboratory methods are clearly described in great detail. The value of the presentation of the clinical material and of the laboratory

procedures is greatly enhanced by well chosen illustrations. As an introduction the author has tried to give the reader a working knowledge of the fundamentals of endocrine physiology. This is a wide field to cover and, at least in the opinion of this reviewer, the author has not reached his goal as a few quotations chosen at random may show. The statement on page 246 that epinephrine "acts on the sympathetic nervous system and all organs and tissues innervated by it" is hardly acceptable on the basis of present day knowledge. On page 236 we read "that it is generally believed that the thyroid hormone raises the level of urea and creatine by promoting the breakdown of both exogenous and endogenous protein." Few workers in the field will agree with this statement as far as creatine is concerned. Notwithstanding the shortcomings of the introductory chapters, the book is to be recommended as a valuable contribution to clinical endocrinology.

W. F.

Medicine and the War. Edited by WILLIAM H. TALIAFERRO. 188 pp. $2.00. The University of Chicago Press, Chicago, 1944.

This booklet consists of a series of lectures on medical topics of particular interest and importance in time of war. Each is written by an authority in his field and with a simplicity and brevity that is outstanding. The authors are all members of the faculty of the Division of Biological Sciences of the University of Chicago. Originally delivered to student audiences, these essays are admirably adapted to reading by educated laymen. The modern concepts of nutrition, the newer amazing chemotherapeutic attack on infections, the physiological problems met with in aviation and the possible transmission of diseases brought about by increased speed of transportation are but some of the topics which should prove informative and delightful reading to the layman as well as to the physician. The reviewer can recommend the volume wholeheartedly and hopes it will have a wide distribution.

J. E. H.

Minor Surgery. By FREDERICK CHRISTOPHER, S.B., M.D., F.A.C.S. 5th ed. 1006 pp. Illus. $10.00. Philadelphia & London, 1944. W. B. Saunders Company.

This is the fifth edition of a standard work that has already found its place in many a surgeon's library. As the owner of an early edition, this reviewer is more than pleased to observe the growth, development and adaptation of the text without the common fault of its appearing to be "patched" over previous models. The objection which many capable surgeons might raise to the very title is covered in the first sentence of the Preface where the author states, "All minor surgery is potentially major surgery, a distinction between the two often being impossible." Actually, it may be observed that the text deals at times with subject matter that might generally be considered to be of the major variety, to wit: compression fractures of the lumbar spine and surgical drainage of empyema. However, in

each instance these subjects fall in well with the material wherein they are grouped and add distinct value to the work.

The author has gone to great pains to make a thorough analysis of the current surgical literature and has made available by direct quotation the "meat" of a great many of these articles in the appropriate places. The sources are widespread and include such authoritative and timely communications as those from the Surgeon General of the U. S. Army and the Official Statements prepared under the auspices of the Division of Medical Sciences of the National Research Council. The section on burns, for instance, covers twenty-six pages, gives a splendid current review of the newer concepts of both general and local treatment, and is well supported by short direct quotations and apt illustrations.

This book is of great value to the surgeon, to the general practitioner who must carry out and understand many lines of surgical therapy, to the medical student, as a reference text, and especially to the surgical house officer for whom it seems especially adapted. Indeed the final chapter entitled "The Surgical Intern" gives a splendid lot of advice which one would wish that every new intern could read and adopt for his own. In this chapter the author from his broad experience advises on such varied subjects as tact toward patients, the importance of being able to address as many nurses as possible by name, and the technic of removing adhesive tape.

S. McL.

Internal Medicine in General Practice. By ROBERT P. MCCOMBS. 694 pp. $7.00. W. B. Saunders Co., Philadelphia, 1943.

The author of this book has recognized the pressing need of the busy practitioner who is out of touch with many of the newer methods of diagnosis and treatment, or confused by the myriads of new medicines, for an authoritative review of the whole subject of medical practice. He has done this remarkably well, concentrating a great store of useful information in a small space, with the result that nothing of real importance seems to have been omitted and only the proven and pertinent facts retained.

Throughout the book the author has stressed the importance of careful diagnostic procedures as well as the dangers of uncritical or symptomatic therapy. From the opening chapter on history-taking and physical examination which emphasizes the short-sightedness of hurried, incomplete studies, to the discussion of psychoneurosis at the end, the subject matter is handled with clarity and conservativism throughout. The chapters on disorders of the gastrointestinal tract, nutritional deficiencies, blood dyscrasias and anemias, rheumatic diseases, and endocrine disorders seem to the reviewer to be particularly well done.

It is to be hoped that the book will have a wide circulation, for it provides good and timely material for all practicing doctors, whose public has become enlightened enough to demand of them better medicine and fewer medicines.

A. M. F.

Notes for the R.M.O. of an Infantry Unit. Oxford War Manuals. By C. P. BLACKER. 77 pp. $1.50. Oxford University Press, New York, 1943.

This is a delightfully simple and interesting summary of a regimental medical officer's experiences in the last war. To some degree, it is a diary and would invoke reminiscences of considerable interest to any medical officer of any country, who served in World War 1. It is, of course, not very deep, nor does it envisage the medical corps as a whole, for the object is only to discuss the problems of a regimental medical officer.

As the author says in his Preface—"This booklet is in no sense intended to be a manual of instructions and duties. These are abundantly set out in the official publications. It is rather a collection of hints and miscellaneous notes which a R.M.O. may find useful when he first assumes his duties with an infantry battalion. It is also largely a record of personal experiences; of mistakes made and lessons learned. In the course of the last three years, new aspects and implications of the R.M.O.'s work have continually been disclosed; routine methods were adapted and improved; and every week, in one way or another, I learned anew it is never too late to learn."

The reviewer fully agrees with the author's summary of his own work.

E. W. B.

Pioneers of Pediatrics. By ABRAHAM LEVINSON. 2nd ed. 119 pp. $2.00. Illus. Frobin Press, New York, 1943.

Dr. Levinson has prepared this revision of "Pioneers of Pediatrics" in a comparatively short time since the publication of the original volume. In this second survey, as well as in the first edition, the policy of excluding the contributions of living American pediatricians, and of all but a few names of contemporary Europeans, was followed. Thus the destruction of hundreds of European pediatric institutions and the passing of many pediatric pioneers has made this revision suitable.

The survey is brief, comprising 119 pages including 28 full page illustrations. The contributions of some 140 "pioneers" are described so that the book is to be considered in no sense comprehensive but rather an abridged review of some of the more outstanding contributors. In-as-much as the specialty of pediatrics is of comparatively recent origin, a large proportion of the contributors, particularly in the earlier pages, are chemists, physicists, general medical practitioners and specialists in a variety of the branches of medicine.

The first 7 chapters deal generally with the progress of pediatrics from the time of the Ebers Papyrus (1500 B.C.) up to and including a part of the 20th century. There follow four special chapters acknowledging in particular authorities on the subject of infant feedings, investigators of alimentary disturbances in infancy, students of the history of pediatrics, and American pioneers of pediatrics.

The book contains numerous and interesting original quotations. It should be a source of entertainment to the pediatrician and a valuable framework for the student interested in pursuing historical aspects of the specialty.

W. G.

Pain Mechanisms. A Physiological Interpretation of Causalgia and Its Related States. By W. K. LIVINGSTON, M.D. 240 pp. $3.75. The MacMillan Co., New York, 1943.

The author is concerned with a number of distressing conditions in which pain is the chief feature. He discusses causalgia, reflex paralysis, chronic low back pain, facial neuralgia, phantom limb pain, etc. He begins with a brief survey of the anatomy of the pain pathways which is followed by a discussion of the physiological mechanisms involved. A short chapter is devoted to the psychology of pain. The clinical section comes next with some case reports and the author's interpretation of the clinical observations.

Dr. Livingston does not claim to reach any final conclusions or to offer any entirely satisfactory method of treatment. He apparently believes that persistent pain may lead to an abnormal state of the central nervous system which may cause widespread disturbances of function including pains distributed throughout the body. He deals with these phenomena in physiological terms and is not sympathetic towards psychological interpretations. Treatment includes the usual surgical procedures such as root section, sympathectomy and resections of the affected nerve trunk but the author apparently finds repeated injections of novocain most effective. These injections he believes interrupt the "vicious circle" and bring about relief. The reviewer cannot claim to be convinced and in most instances clings to the psychological point of view.

F. R. F.